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Inhibition of endothelium-derived hyperpolarizing factor by ascorbate in the bovine eye

A thesis submitted for the degree of Doctor of Philosophy in
Faculty of Biomedical and Life Sciences at the University of

Glasgow

By

Alister J McNeish[©]



Division of Neuroscience and Biomedical Systems

West Medical Building

University of Glasgow

Glasgow G12 8QQ

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Summary



1. The aim of this study was to characterise vasodilator responses in the perfused ciliary vascular bed of the bovine eye
2. When bovine eyes were perfused at a constant rate of 2.5 ml min^{-1} , infusion of the powerful vasodilator, papaverine ($150 \text{ } \mu\text{M}$), produced a very small reduction in perfusion pressure. Under the same conditions, the nitric oxide synthase inhibitor, L-NAME ($100 \text{ } \mu\text{M}$), had no effect but the inhibitor of soluble guanylate cyclase, ODQ ($10 \text{ } \mu\text{M}$), produced a small vasoconstrictor response. These results indicate that there is a small component of intrinsic (myogenic) tone that may be suppressed by a basal release of nitric oxide.
3. In the bovine eye, vasodilatation to acetylcholine or bradykinin was unaffected by L-NAME ($100 \text{ } \mu\text{M}$), or the cyclo-oxygenase inhibitor, flurbiprofen ($30 \text{ } \mu\text{M}$), but was significantly attenuated following treatment with a high concentration of KCl (30 mM), or by damaging the endothelium with the detergent, CHAPS (0.3% , 2 min). Thus agonist-induced vasodilatation is not mediated by nitric oxide or prostacyclin but involves a K^+ conductance and is endothelium-dependent.
4. Acetylcholine-induced vasodilatation in the bovine eye was unaffected by glibenclamide ($10 \text{ } \mu\text{M}$), an inhibitor of ATP-sensitive K^+ channels (K_{ATP}), but was significantly attenuated by TEA (10 mM), a non-selective inhibitor of K^+ channels. The blockade of vasodilatation by TEA but not glibenclamide could indicate that a calcium-sensitive K^+ channel is involved in the response.
5. The small conductance calcium-sensitive K^+ channel (SK_{Ca}) inhibitor, apamin (100 nM), and the large conductance calcium-sensitive K^+ channel (BK_{Ca}) inhibitor, iberiotoxin (50 nM), had no significant effect on acetylcholine-induced vasodilatation. In contrast, the intermediate (IK_{Ca})/large conductance calcium-sensitive K^+ channel inhibitor, charybdotoxin (50 nM), powerfully blocked these vasodilator responses, and

uncovered a vasoconstrictor response. Thus, vasodilator responses appear to involve the opening of IK_{Ca} channels.

6. The combination of apamin (100 nM) with a sub-threshold concentration of charybdotoxin (10 nM) significantly attenuated acetylcholine-induced vasodilatation, but the combination of apamin (100 nM) with iberiotoxin (50 nM) had no effect. This profile of blockade is consistent with the vasodilator responses being mediated by endothelium-derived hyperpolarizing factor (EDHF).

7. Ascorbate is known to protect nitric oxide dependent vasodilatation under conditions of oxidant stress, however, EDHF-mediated vasodilator responses induced by acetylcholine or bradykinin were powerfully blocked when ascorbate (50 μ M, 120 min) was included in the perfusion medium; with acetylcholine a normally masked muscarinic vasoconstrictor response was also uncovered. These results indicate that, ascorbate at a physiologically relevant concentration, can inhibit EDHF-mediated vasodilations.

8. The blockade of EDHF-mediated vasodilatation by ascorbate was time-dependent (maximum blockade at 120 min) and concentration-dependent (10-150 μ M). Thus, the blocking action of ascorbate has a slow onset and occurs concentrations across the normal plasma concentration range (10 –150 μ M).

9. The ability of ascorbate to block EDHF-mediated vasodilatation in the bovine eye is likely to result from its reducing properties, since this action was mimicked by two other reducing agents, namely, N-acetyl-L-cysteine (1 mM) and dithiothreitol (100 μ M), but not by the redox-inactive analogue, dehydroascorbate (50 μ M).

10. In the bovine eye, vasodilations induced by the K_{ATP} opener, levcromakalim (100 pmol-30 nmol), or the nitric oxide donor, glyceryl trinitrate (10 nmol), were completely unaffected by the infusion of ascorbate (50 μ M). Furthermore, the L-NAME induced

vasoconstrictor response in the presence of U46619 (~200 nM) was unaffected by infusion of ascorbate (50 μ M). Thus, the blockade of EDHF-mediated vasodilatation by ascorbate is highly selective and does not result from non-selective damage of the endothelium as basal release of nitric oxide is unaffected.

11. Ascorbate (50 μ M) also blocked acetylcholine-induced, EDHF-mediated vasodilator responses in the rat mesenteric arterial bed in a time-dependent manner (maximum blockade at 180 min), indicating that this effect of ascorbate is not exclusive to the eye.

12. In rings of porcine coronary artery the EDHF-mediated vasodilatation induced by bradykinin (0.1-30 nM) was unaffected by charybdotoxin (100 nM), slightly inhibited by apamin (100 nM), but virtually abolished by the combination of the two blockers. In the presence of ascorbate (150 μ M) this EDHF-mediated vasodilatation induced by bradykinin was unaffected. Furthermore, ascorbate failed to cause further inhibition of vasodilatation when combined with either charybdotoxin or apamin alone, or in combination. These results indicate that ascorbate cannot block EDHF-mediated vasodilatation in all vascular preparations.

13. Interfering with the ability of bovine eyes to accumulate ascorbate, either by flushing the anterior and posterior chambers or by draining these chambers and the vitreous cavity, did not affect the ability of ascorbate (50 μ M) to inhibit acetylcholine (10 nmol)-induced EDHF-mediated vasodilations. Thus, the ascorbate-induced inhibition of EDHF-mediated vasodilator responses is independent of the eyes ability to accumulate ascorbate.

14. These results indicate that, in the bovine eye there is basal release of nitric oxide, which suppresses perfusion pressure but does not contribute to agonist-induced vasodilatation. However, acetylcholine- and bradykinin-induced, endothelium-dependent vasodilations in the perfused ciliary vascular bed of the bovine eye are

mediated entirely by EDHF. These EDHF-mediated vasodilator responses are inhibited by the antioxidant, ascorbate, at concentrations similar to those normally found in the blood plasma. An antioxidant mechanism accounts for the blockade of EDHF-mediated vasodilatation by ascorbate and this blockade is highly selective. Ascorbate also blocks EDHF-mediated vasodilatation in the perfused rat mesentery, however, results from the porcine coronary artery indicate that ascorbate does not block EDHF in all vascular preparations.

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Abbreviations



1-EBIO	1-ethyl-2-benzimidazolinone
1400W	N-(3-(aminomethyl)benzyl)acetamide
BK _{Ca}	large conductance calcium-sensitive K ⁺ channel
COX	cyclo-oxygenase
CHAPS	3-[(cholamidopropyl)dimethyl-ammonio]1-propanesulfonate
ChTx	charybdotoxin
DCEBIO	5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one
DTT	dithiothreitol
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor (nitric oxide)
FBP	flurbiprofen
HMTA	hexamethylenetetramine
IbTx	iberiotoxin
IK _{Ca}	intermediate conductance calcium-sensitive K ⁺ channel
K _{ATP}	ATP-sensitive K ⁺ channel
L-NAME	N ^G -nitro-L-arginine methyl ester
NAC	N-acetyl-L-cysteine
NO	nitric oxide
NOS	nitric oxide synthase
NS1619	1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one
ODQ	1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one
SK _{Ca}	small conductance calcium-dependent K ⁺ channel
TEA	tetraethylammonium
U46619	9,11-dideoxy-11 α ,9 α -epoxy-methanoprostaglandin F _{2α}

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Declaration



I declare that this thesis has been composed by myself and is a record of my work performed by myself (except where indicated). It has not been previously submitted for a higher degree.

The research was carried out in the institute of biomedical and life sciences in the division of neuroscience and biomedical systems, University of Glasgow, under the supervision of Professor William Martin and Dr William Wilson.

Alister McNeish

November 2002

Chapter 1

Introduction



1.1 The bovine eye

1.1.1 Anatomy of the bovine eye

The eye is a fluid-filled sphere, the globe of which is essentially made up of three different layers: the outermost layer consists of the cornea and the sclera and its function is mainly to provide structural strength to the eye and, as such, it also acts as a protective barrier; the middle layer, the uvea, consists of the highly vascular choroid, the ciliary body and the iris; and the innermost layer of the eye is the retina, which is the light-sensing layer containing all the neural cell types essential for vision (Figure 1.1).

There are three fluid-filled chambers within the eye: the anterior chamber, the posterior chamber and the vitreous cavity (Figure 1.1). The anterior and posterior chambers are filled with aqueous humour, a clear, colourless liquid that supplies nutrients to the avascular structures of the eye such as the cornea and lens (Millar & Kaufman, 1995)(see Section 1.1.3). The anterior and posterior chambers are continuous via the pupil of the eye. The vitreous cavity is located behind the lens and ciliary body and is the largest of the three chambers; it is filled with a clear jelly-like substance, the vitreous humour.

The cornea is the transparent avascular structure that covers the anterior surface of the globe (Figure 1.1). The cornea consists of four layers: the epithelium, stroma, Descemet's membrane and endothelium and is the major refractive component of the eye. Another refractive component of the eye is the lens, a transparent and crystalline structure; it is located immediately posterior to the iris (Figure 1.1) and functions to focus light on to the retina. The lens is supported by the suspensory ligaments, which are attached to the ciliary body (Prince *et al.*, 1960).

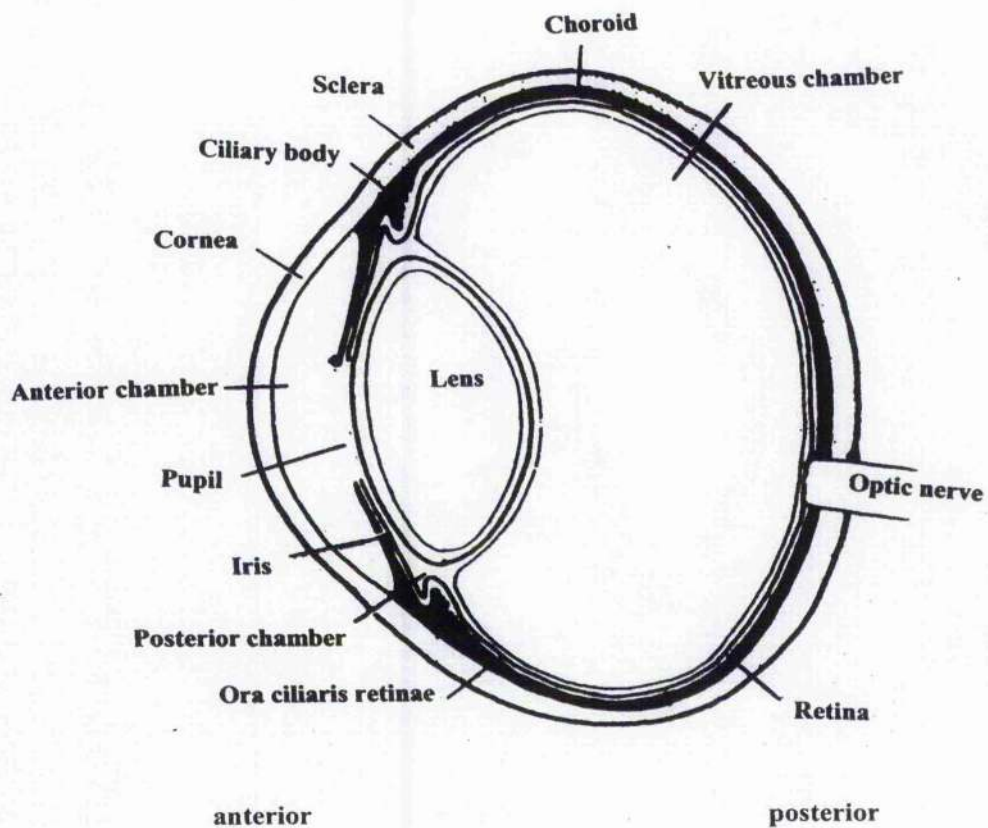


Figure 1.1 Schematic diagram of the vertical cross section of the bovine eye, showing the gross anatomy of the globe including: the three layers of the globe (retina, choroid and sclera), the ocular chambers, the iris, the ciliary body and the lens.

The iris is the anterior portion of the uvea and is often darkly pigmented; it is located between the cornea and the lens and forms the boundary between the anterior and posterior chambers (Figure 1.1). In the bovine eye the iris forms an oval pupil, which is oriented in the horizontal plane, but becomes almost circular when dilated (Prince *et al.*, 1960). This is in contrast to the circular pupil of human eyes. Indeed, the anatomy of the eye varies greatly amongst mammalian species and anatomical knowledge of the human eye may lead to misinterpretation of the anatomical arrangement of the bovine eye; therefore Table 1.1 displays a list of some major anatomical differences between bovine and human eyes.

The ciliary body extends from the termination of the retina (ora ciliaris retinae; Figure 1.1) to the root of the iris (Prince *et al.*, 1960). The majority of the ciliary body's mass is the ciliary muscle, arranged into three orientations: radial, circular and longitudinal (Millar & Kaufman, 1995). The ciliary muscle functions to stretch the lens in order to focus light on the retina. However, there is little evidence for functional activity of the ciliary muscle in the bovine eye (Prince *et al.*, 1960); perhaps indicating that, like other ungulates, the bovine eye has a limited ability for accommodation (Diescem, 1975). A number of projections, termed ciliary processes, radiate from the ciliary body into the posterior chamber. The anatomy of the ciliary body is described in more detail in Section 1.1.3.1.

1.1.2 Vascular system

In most mammalian eyes, there are two distinct vascular systems to supply the anterior and posterior of the eye; blood is usually supplied to the retina by retinal and short posterior ciliary arteries, and to the anterior eye from long posterior ciliary arteries and

Anatomical feature	Human eye	Bovine eye
Globe dimensions (mm)	21-26 (anterior-posterior) 23-25 (diameter) ⁵	28-30 (anterior-posterior) 30 (diameter) ³
Main arterial blood supply	Internal ophthalmic artery ³	External ophthalmic artery (via ciliary artery) ³
Lens dimensions (mm)	~4 (anterior-posterior) ~10 (diameter) ⁵	~13 (anterior-posterior) ~19 (diameter) ³
Lens Volume (ml)	0.2 ⁵	2.2 ⁴
End of retina/start of ciliary body	Ora serrata ⁵	Ora ciliaris retinae ³
Ciliary muscle	Well developed ^{3,6}	Poorly developed ³
Accommodation	Extensive ^{3,5,6}	Limited ³
Scleral spur	Present ⁵	Absent ³
Anterior chamber volume (ml)	0.35 ⁵	1.7 ⁴
Ciliary processes	~70 ⁵	90-110 ³
Pupil	Circular ^{3,6}	Oval ³
Corneal dimensions (mm)	~11.7 (horizontal) ⁵ ~10.6 (vertical) ~1 (thickness)	27-34.5 (horizontal) ^{1,2} 20-30.5 (vertical) 0.7-1.5 (thickness)
Canal of Schlemm	Present ⁵	Absent ³

Table 1.1 Some anatomical differences between the bovine and human eye, adapted from ¹(Doughty, 1997) ²(Doughty *et al.*, 1995) ³(Prince *et al.*, 1960) ⁴(Shahidullah, 1994) ⁵(Tripathi & Tripathi, 1984) ⁶(Williams & Warwick, 1980).

anterior ciliary arteries (Alm, 1992). In the bovine eye, the majority of the blood is supplied by the ciliary arteries (Figure 1.2)(Prince *et al.*, 1960), which branch off from the *external* ophthalmic artery; this is in contrast to human eyes where most of the blood is supplied by the *internal* ophthalmic artery (Table 1.1). In the bovine eye, many of the arteries supplying the extraocular muscles are formed from the external ophthalmic artery, and there are usually several to each extraocular muscle. The anterior ciliary arteries are branches of these muscular arteries and enter the sclera near the root of the iris (Prince *et al.*, 1960).

In the bovine eye the ciliary artery is aligned with the optic nerve; here it divides into the large medial and the smaller lateral ciliary artery, both running in parallel, loosely attached to the optic nerve (Figure 1.2). These arteries bifurcate before entering the globe forming many short posterior ciliary arteries. Some of the small posterior ciliary arteries enter the globe at the same point as the optic nerve and of these, some branch to form the retinal arteries (Prince *et al.*, 1960). The other short posterior ciliary arteries enter the sclera at varying distances from the optic nerve, some running along the choroid towards the iris and ciliary body forming branches to the surrounding tissues.

The large medial ciliary artery also bifurcates to form two long posterior ciliary arteries, both of which travel along the globe of the eye, one along the medial side, and the other along the lateral side (Figure 1.2). The long posterior ciliary arteries enter the sclera posterior to the equator and travel along the horizontal plane on the internal surface of the choroid, in the vitreous chamber, to the root of the iris. Here they anastomose with the anterior ciliary arteries and form the major arterial circle of the iris (Figure 1.3) at the periphery of the iris, thus supplying blood to the ciliary body and iris (Caprioli,

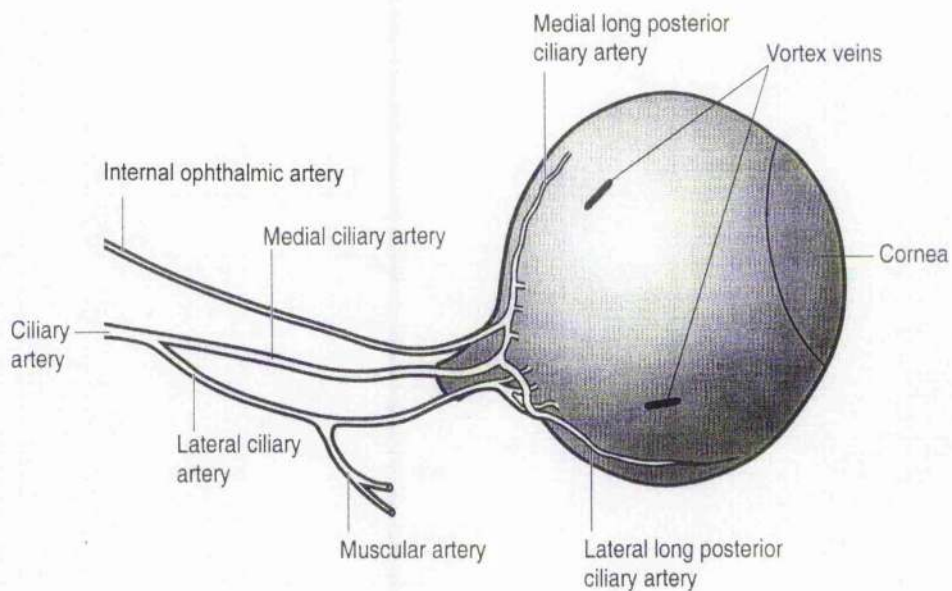


Figure 1.2 Schematic diagram showing the major arterial blood supply and the vortex veins of the bovine eye. Most of the blood is supplied *via* the ciliary artery, with a minor contribution from the internal ophthalmic artery. Blood is supplied to the anterior structures of the eye such as the ciliary body and iris mostly by the long posterior ciliary arteries. This blood is returned to the systemic circulation by the four vortex veins located around the equator of the globe. Adapted from (Prince *et al.*, 1960)

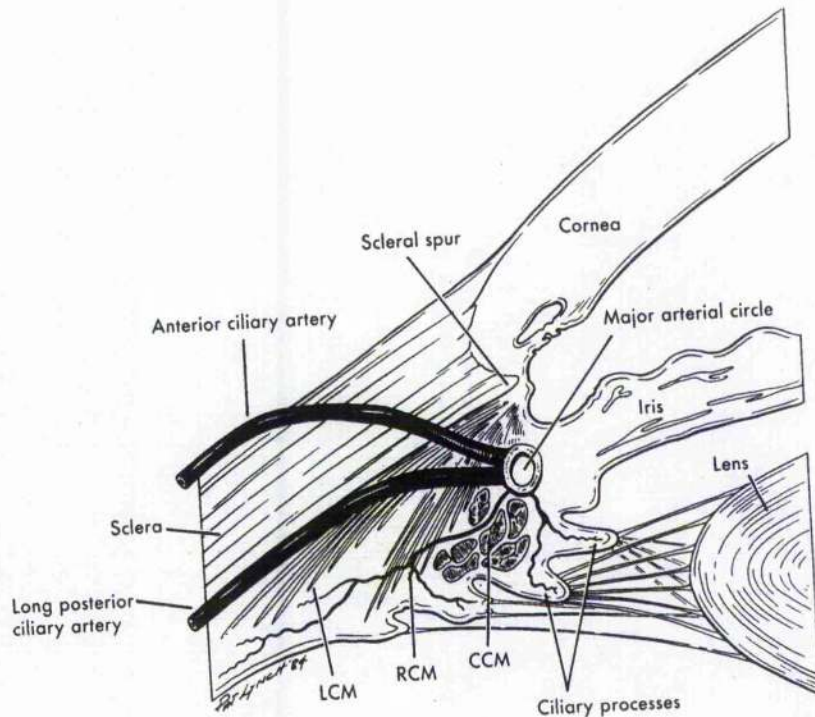


Figure 1.3 Schematic diagram of the ciliary body of the human eye, showing the components of ciliary muscle and the major arterial circle of the iris and the blood supply to the ciliary processes. LCM, longitudinal ciliary muscle; RCM, radial ciliary muscle; and CCM, circular ciliary muscle. N.B. Although the anatomy of the bovine ciliary body is similar to that of the human there are some major differences (see Table 1.1); for example there is no scleral spur in the bovine eye. In addition, in the bovine eye, the long posterior ciliary arteries are much larger than the anterior ciliary arteries and as such supply most of the blood to the major arterial circle. Adapted from (Caprioli, 1992).

1992); the major arterial circle of the iris is well developed in bovine eyes (Prince *et al.*, 1960).

The venous system of the eye is also complex and, as with the arterial system, can vary significantly among species. In most mammalian eyes, including the bovine, blood returning from the retina drains through the ciliary veins, which pass along the optic nerve and eventually drain into the internal maxillary vein (Prince *et al.*, 1960). The blood supplied to the choroid *via* the small posterior ciliary arteries and that supplied to the iris and ciliary body by the long posterior and the anterior ciliary arteries is drained by the vortex veins (Prince *et al.*, 1960). There are four vortex veins in the bovine eye, which leave the eye in the region of the equator and are fairly evenly spaced around the globe (Figure 1.2; Prince *et al.*, 1960).

1.1.3 The ciliary body, aqueous humour formation and dynamics

1.1.3.1 Anatomy of the ciliary body and ciliary processes

As described in section 1.1.1, the ciliary body forms a muscular ring along the inner wall of the eye posterior to the iris, anterior to the retina, and is involved in stretching the lens to focus light on the retina. The ciliary body is also the tissue responsible for the formation of aqueous humour, which is the source of nutrients to the avascular structures of the eye.

A large number of villus-like structures project from the inner surface of the ciliary body into the posterior chamber of the eye. These are the ciliary processes and they are the locus of aqueous humour formation (Figure 1.3; Millar & Kaufman, 1995). In the bovine eye there are about 90-110 of these ciliary processes (Prince *et al.*, 1960) and

their dimensions can vary. In the human eye, for example, the ciliary processes average 2 mm in length and 0.5 mm width and 1 mm high (Millar & Kaufman, 1995). The processes have a convoluted shape, they consist of capillaries surrounded by connective tissue (the stroma) and are covered by a double epithelial layer, the ciliary epithelium (Millar & Kaufman, 1995).

Blood is supplied to the ciliary processes from the major arterial circle of the iris *via* radial ciliary arteries. The blood is then drained *via* ciliary venules to the vortex veins (Millar & Kaufman, 1995). The capillaries of the ciliary processes are large, thin walled and highly fenestrated; therefore they are highly permeable. Combined with the convoluted shape, this provides the ciliary processes with a large, porous surface area from which to secrete aqueous humour. The capillaries are surrounded by the stroma, which consists mainly of loose connective tissue and collagen. A basement membrane separates the stroma from the ciliary epithelium (Millar & Kaufman, 1995).

The ciliary epithelium consists of a double layer of cells, one pigmented and the other non-pigmented and represent the continuation of the retinal pigmented epithelium and the retinal neuroepithelium, respectively (Bill, 1975). A striking feature of the ciliary epithelium is that the apical surfaces of the pigmented and non-pigmented ciliary epithelial cells face each other (Figure 1.4). The basolateral surface of the pigmented epithelium is attached to the basement membrane, whereas the basolateral surface of the non-pigmented epithelial cells bounds the posterior chamber (Figure 1.4). Prominent features of the ciliary epithelial cells include interdigitations between the lateral surfaces of neighbouring cells and basal infoldings on the non-pigmented cells (Caprioli, 1992);

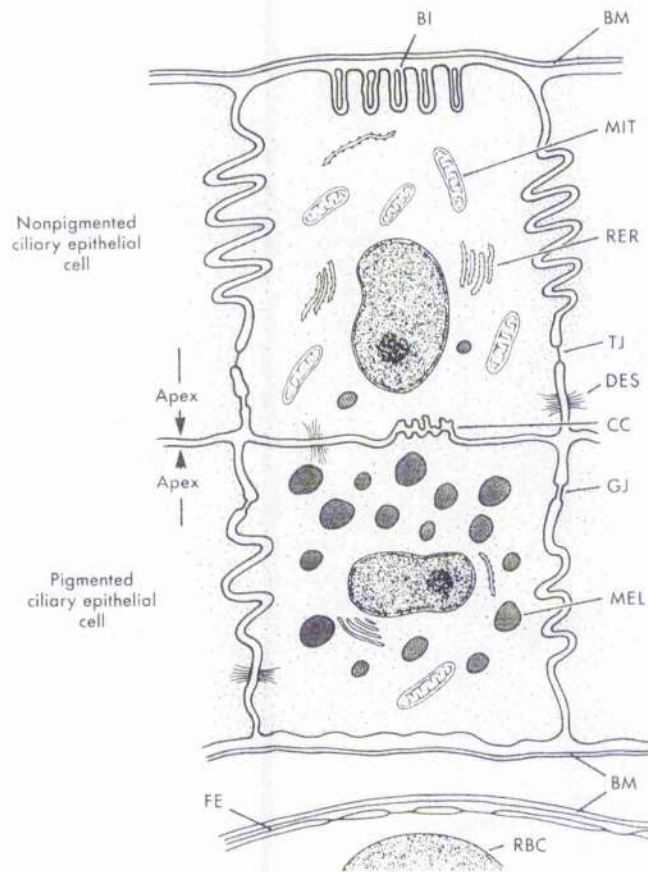


Figure 1.4 Schematic diagram of the pigmented and non-pigmented epithelial cells. The apices of the cells face each other and they form the double-layered ciliary epithelium surrounding the ciliary processes. The basolateral side of the non-pigmented cells is in the posterior chamber, whereas the basolateral surface of the pigmented cells faces the stroma of the ciliary processes. BI, basal infoldings; BM, basement membrane; CC, ciliary channels; DES, desmosomes; FE, fenestrated capillary endothelium; GJ, gap junction; MEL, melanosome; MIT, mitochondrion; RBC, red blood cell; RER, rough endoplasmic reticulum; and TJ, tight junction. Adapted from (Caprioli, 1992).

both of these features are characteristic of epithelial cells involved in secretion (Bill, 1975). Furthermore, there are also tight junctions between lateral interdigitations of the non-pigmented cells. These exclude large molecules and form an integral part of the blood-aqueous barrier (Millar & Kaufman, 1995).

1.1.3.2 Aqueous humour dynamics

As stated in Section 1.1.3.1, the ciliary processes are the sites of aqueous humour production. The aqueous humour is a clear liquid, similar in chemical composition to blood plasma (Table 1.2), which supplies the avascular structures of the eye with nutrients and oxygen, while removing metabolites and carbon dioxide. The aqueous humour is continually formed and drained (Millar & Kaufman, 1995). In brief, aqueous humour is made ultimately from the blood plasma in the capillaries of the ciliary processes, by a process involving passive diffusion, ultrafiltration and active transport; indeed, active transport of solutes across the ciliary epithelium is thought to be the primary process in aqueous humour formation (Caprioli, 1992).

Once the aqueous humour is formed it flows through the posterior and anterior chambers and drains into the bloodstream via two main pathways. The first and major route is through the trabecular meshwork, a network of connective tissue covered by endothelial cells (Fatt & Weissman, 1992), which in primates, leads to the canal of Schlemm; aqueous humour drained from the trabecular pathway enters the aqueous veins from where it is returned to the systemic circulation via the vortex veins. The second pathway may account for up to 20% of aqueous drainage and is thought to be simple diffusion of aqueous through the ciliary muscle and sclera into the orbit where the lymphatic system absorbs it. This pathway is often termed the uveoscleral or trans-scleral route (Fatt & Weissman, 1992).

Substance	Bovine AH	Human AH	Plasma
<i>Electrolytes</i>			
Na ⁺ (mM)	143.8-149.5 ³	142 ⁶	130-145 ^{2,4}
K ⁺ (mM)	4.5-7.1 ^{1,3}	4 ⁶	3.5-5.0 ^{2,4}
Cl ⁻ (mM)	116.3-124 ³	131-136 ²	92-125 ^{2,4}
HCO ₃ ⁻ (mM)	36 ⁶	20 ²	24-30 ^{2,4}
<i>Organic substances</i>			
Ascorbate (mM)	2 ⁷	1.0-1.1 ^{2,7}	0.04-0.06 ^{2,4}
Lactate (mM)	7.6-8.2 ¹	4.5 ^{2,4}	0.5-1.9 ^{2,4}
Glucose (mM)	2.2 ¹	2.8-3.9 ⁶	5.6-6.4 ^{2,4}
Protein (mg ml ⁻¹)	0.2-0.8 ^{5,7}	0.3-0.7 ²	60-70 ^{2,4}

Table 1.2 The composition of some common electrolytes and organic solutes in the bovine and human aqueous humour (AH) and blood plasma (from a range of species). Adapted from ¹(Bito L.Z. & Salvador E.V, 1970) ²(Caprioli, 1992) ³(Carter *et al.*, 1973) ⁴(Cole, 1984) ⁵(Johnson *et al.*, 1986) ⁶(Shahidullah, 1994) and ⁷(Davson, 1969).

As described above, the composition of the aqueous humour is similar to that of blood plasma (Table 1.2). However, there are some major differences in their composition. For example, the blood aqueous barrier largely excludes plasma proteins, thus limiting levels of protein to about 1% of that normally seen in plasma. Indeed, elevated levels of protein in the aqueous humour is indicative of a breakdown of the blood aqueous barrier (Caprioli, 1992). The relative composition of proteins in the aqueous humour is also different to blood plasma. Low-molecular weight proteins such as albumin are present in aqueous humour and plasma, whereas larger proteins such as immunoglobulins are normally only found in plasma, and are absent from the aqueous under normal conditions (Caprioli, 1992).

In contrast to the low levels of protein, the endogenous low-molecular weight antioxidant, ascorbate (Halliwell & Gutteridge, 1989), is found at higher concentrations in the aqueous humour than in the blood plasma (Table 1.2). The normal plasma concentration of ascorbate in humans is $46 \pm 8 \mu\text{M}$ (range 30–150 μM ; Keaney & Vita, 1995; Levine *et al.*, 1996) whereas the concentration of this antioxidant in aqueous humour is around 1 mM in humans and a wide range of animal species (Caprioli, 1992; Davson, 1980; Halliwell & Gutteridge, 1989). There is much speculation over why ascorbate is so concentrated in the aqueous humour, but since it avidly scavenges the superoxide anion (Som *et al.*, 1983), it may be to compensate for the particularly low levels of superoxide dismutase found in the eye (Halliwell & Gutteridge, 1989).

1.1.3.3 Aqueous humour, intraocular pressure and disease

The intraocular pressure (IOP) is essential for maintaining the internal structures and shape of the eye. IOP is dictated by the rates of formation and drainage of aqueous

humour from the eye and the pressure in the episcleral veins to which it drains. A change in aqueous humour formation, or a change in resistance to drainage will cause a proportionate change in IOP. The normal IOP in the human eye is approximately 15 mmHg (range, 10.5-20.5 mmHg, for healthy eyes (Davson, 1984).

The Glaucomas are a group of diseases that lead to progressive damage of the retina and optic nerve head leading to loss of peripheral vision and eventually blindness (Leopold & Duzmann, 1986; Serle, 1994). A high IOP (>21 mmHg; Fatt & Weissman, 1992) is a well known risk factor for the development of glaucoma, although in low tension glaucoma the pathological changes to the field of vision occur within normal IOP boundaries. Regardless, in some forms of the disease, such as open-angle and closed-angle glaucoma, the increase in the IOP is due to decreased drainage of aqueous (although increased production may contribute to open angle glaucoma; Fatt & Weissman, 1992). Indeed, many of the current treatments for glaucoma work by decreasing the resistance to drainage of aqueous from the eye, thereby lowering IOP. For example, topically applied prostaglandin $F_{2\alpha}$ analogues, such as latanoprost (Willis *et al.*, 2002) increase uveoscleral drainage, whereas cholinergic agents, such as pilocarpine, increase drainage through the trabecular meshwork (Hurvitz *et al.*, 1991). An alternative treatment for glaucoma is to decrease aqueous humour formation (and therefore IOP) using either an α_2 -adrenoceptor agonist, such as apraclonidine, or β -adrenoceptor antagonists such as timolol, which bind to receptors on the ciliary epithelium (Hurvitz *et al.*, 1991).

1.2 Endothelium-dependent vasodilator mechanisms

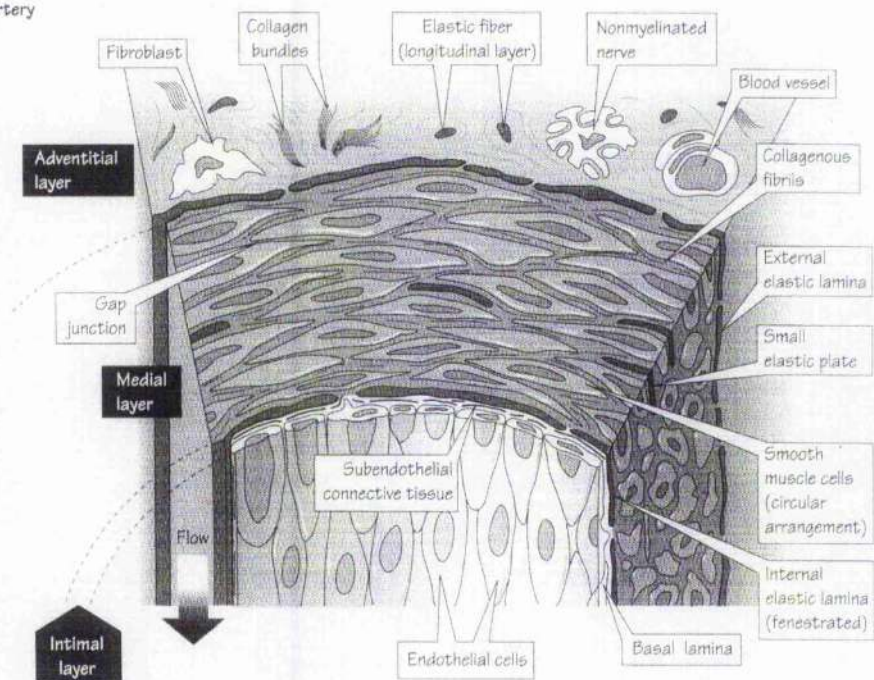
1.2.1 Anatomy of arteries

The arteries are responsible for transport of blood from the heart to the tissues of the body. The aorta is the largest artery and along with its major branches is termed an elastic artery, these arteries distend during systole and recoil during diastole in order to dampen the pulse wave of the heart beat, helping to even out the flow of blood. These branch to form muscular arteries, which have thicker walls to prevent collapse when joints bend. In turn, the muscular arteries branch to form resistance vessels and even smaller resistance arterioles, which present the largest resistance to flow of blood and are largely responsible for generating blood pressure (Aaronson *et al.*, 1999; Berne & Levy, 2000).

Regardless of the type of artery, they all have a similar anatomy. Arteries are essentially tubes of smooth muscle consisting of three layers (Figure 1.5a): the intimal layer consisting of a single layer of endothelial cells; the thick medial layer consisting of smooth muscle cells; and the outer adventitial layer, which is mainly connective tissue (Aaronson *et al.*, 1999).

The intimal, endothelial cell layer lines the vascular lumen and the chambers of the heart. The endothelial cells are joined by tight junctions and can act as a barrier to restrict movement of large molecules (Aaronson *et al.*, 1999). During the inflammatory response there is vasodilatation and an increase of blood flow, the endothelial barrier is “opened” increasing vascular permeability, leading to exudation of fluid and inflammation. Furthermore, atherosclerosis is associated with increased endothelial cell permeability and turnover, abnormally activated macrophages release cytokines causing

(a) Muscular artery



(b) Smooth muscle cell ultrastructure

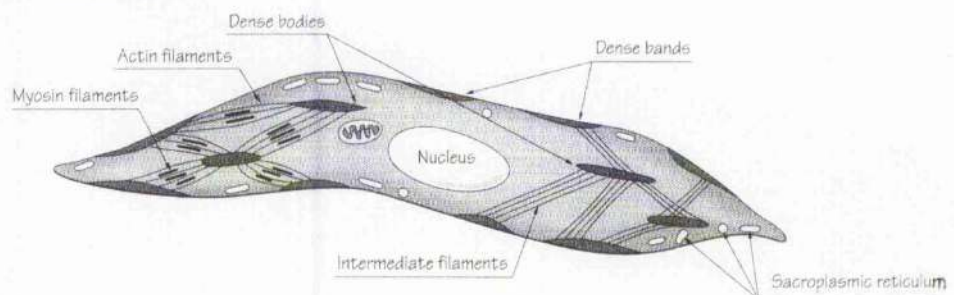


Figure 1.5 Schematic diagram of (a) the major anatomical features of a muscular artery, including the main components of the intimal, medial and adventitial layers, and (b) a single vascular smooth muscle cell. Taken from (Aaronson *et al.*, 1999)©.

an inflammatory response that leads to formation of a plaque. The endothelial cells have other functions, for example they are involved in processes such as haemostasis. In haemostasis damage of the endothelium exposes collagen to the blood, activating platelets, which leads to vasoconstriction and formation of a blood clot. However, under normal conditions endothelial cells also release nitric oxide and prostacyclin, which prevent platelet aggregation. They also release thrombomodulin, which starts a chemical cascade eventually leading to fibrinolysis, the breakdown of a blood clot. The endothelial cells are also involved in controlling vascular tone and can release a number of vasoconstrictor and vasodilator agents which can act on the underlying smooth muscle layer. For example, angiotensin converting enzyme (ACE), found on the plasma membrane of endothelial cells, converts angiotensin I to form angiotensin II, a potent vasoconstrictor (Rang *et al.*, 1999). ACE also functions to inactivate the potent vasodilator, bradykinin (Rang *et al.*, 1999). The vasodilator mechanisms of endothelial cells are discussed in sections 1.2.2, 1.2.3 and 1.2.4.

The medial layer is separated from the endothelial cells by the internal elastic lamina (Figure 1a) and consists mainly of smooth muscle cells in an extracellular matrix of collagen. The smooth muscle cells have a shape similar to an irregular cylinder (Figure 1.5b), or spindle, and are arranged in a circular manner in order to oppose the radial force of pressure and so that when they contract, the lumen diameter of the artery is decreased (Aaronson *et al.*, 1999).

The outer, adventitial layer is separated from the medial layer by the external elastic lamina, the collagen-rich connective tissue that makes up its bulk and supports nerves and fibroblasts. In larger arteries, the adventitial layer may also contain small blood

vessels (the vasa vasorum) to supply the medial layer with nutrients. Most arteries are innervated with sympathetic fibres that release noradrenaline to cause vasoconstriction. Some specialised arteries may also be innervated by parasympathetic nerve fibres, which release a variety of transmitters such as CGRP in the rat mesenteric artery (Kawasaki *et al.*, 1988) (sensory nerves) or nitric oxide in penile (Burnett *et al.*, 1992) and cerebral arteries (Bredt *et al.*, 1990), to cause vasodilatation.

1.2.2 Nitric oxide

1.2.2.1 Characterisation of endothelium-derived relaxing factor as nitric oxide.

Furchgott and Zawadzki (1980) first demonstrated that the acetylcholine-induced relaxation of rabbit aorta was dependent upon the presence of the endothelium and that a diffusible substance released by the endothelium was responsible for the relaxation. Vasodilator responses mediated by this endothelium-derived relaxing factor (EDRF) were later demonstrated in almost every vascular preparation from a wide variety of species to many different chemical stimuli (e.g. bradykinin, calcium ionophore A23187 and substance P (for review see: Moncada *et al.*, 1991). EDRF-mediated vasodilatation was also shown to be stimulated by blood flow (Rubyani *et al.*, 1986). Furthermore, EDRF was shown to be released spontaneously, i.e. under unstimulated conditions, in some preparations (Martin *et al.*, 1986).

It was soon established that EDRF was a substance with a half-life of a few seconds (Cocks *et al.*, 1985; Griffith *et al.*, 1984) and that its effects were mediated through stimulation of soluble guanylate cyclase in smooth muscle cells to raise cyclic GMP levels (Rapoport & Murad, 1983); raising cyclic GMP levels which activates protein kinase G which causes relaxation of vascular smooth muscle by lowering the

intracellular calcium levels and phosphorylating myosin light-chain kinase (MLCK; Collins *et al.*, 1986). Indeed, the actions of EDRF were attenuated by inhibitors of guanylate cyclase such as methylene blue (Martin *et al.*, 1985), or the more recently introduced ODQ (Garthwaite *et al.*, 1995). The EDRF was also shown to inhibit platelet aggregation and cause disaggregation of aggregated platelets (Azuma *et al.*, 1986; Radomski *et al.*, 1987). In 1986 it was concluded that due to the similar pharmacological profiles of EDRF and nitric oxide, EDRF might indeed be nitric oxide (Furchgott, 1988; Ignarro *et al.*, 1988). Experiments comparing the effects of EDRF and nitric oxide on vascular strips and on platelets, found that the two were indistinguishable (Moncada *et al.*, 1988; Palmer *et al.*, 1987). Thus it is widely accepted that EDRF is nitric oxide. Figure 1.6 shows an overview of the nitric oxide pathway.

1.2.2.2 Synthesis of nitric oxide

Nitric oxide is enzymatically synthesised by nitric oxide synthase, a dimerised enzyme with both reductase and oxidase functions (Hobbs *et al.*, 1999). Nitric oxide synthase cleaves the terminal guanidine nitrogen group of the amino acid, L-arginine, in the presence of O₂, in a five-electron oxidation to form nitric oxide and L-citrulline at an active site containing a heme-moiety. Nitric oxide synthase also requires several co-factors including: Ca²⁺, which is involved in activating the enzyme; calmodulin, which in the presence of Ca²⁺ binds to the enzyme tightly and is involved in electron transfer between the reductase and oxidase domains of the enzyme (Abu-Soud & Stuehr, 1993); NADPH, to supply the electrons for the oxidation of L-arginine; flavin adenine dinucleotide is involved in electron transfer from NADPH to the heme site (Stuehr & Ghosh, 2000), flavin mononucleotide is also involved in this process; and

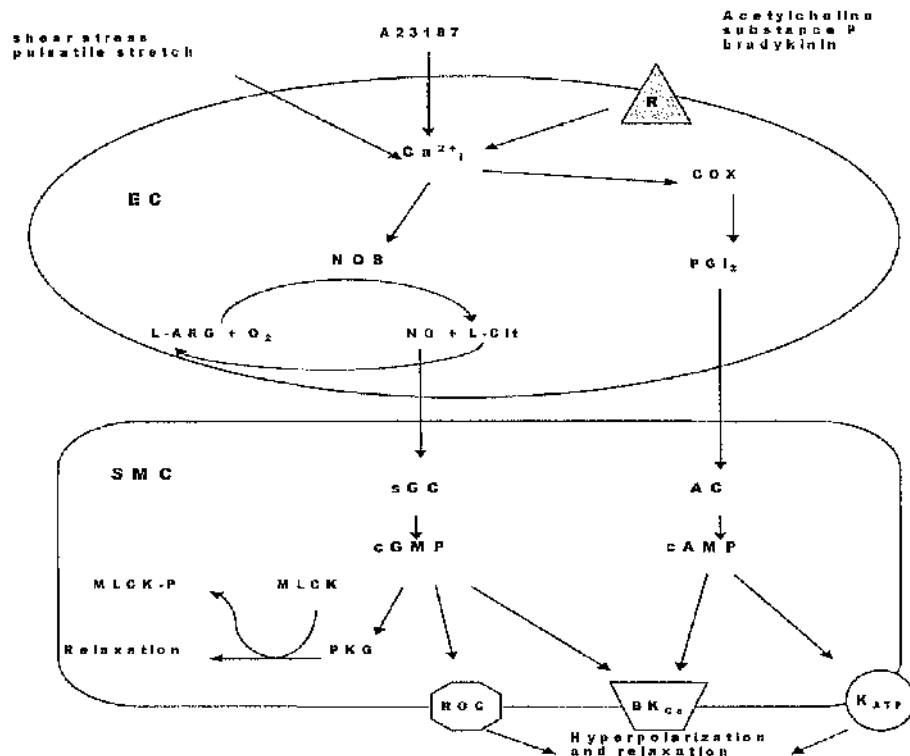


Figure 1.6 Schematic diagram showing the synthesis, release and cellular targets of nitric oxide (NO) and prostacyclin (PGI₂) (see text for details). NO is synthesised in endothelial cells (EC) by NO synthase (NOS). NO diffuses to smooth muscle cells where it activates soluble guanylate cyclase (sGC), which produces cyclic GMP (cGMP). cGMP causes relaxation by activating protein kinase G (PKG), thus inhibiting myosin light chain kinase, by phosphorylating it (MLCK-P); and inhibition of receptor-operated Ca²⁺ channels (ROC) or activation of Ca²⁺-ATPase. PGI₂ produced by cyclooxygenase (COX) stimulates adenylate cyclase (AC), producing cyclic AMP which activates protein kinase A (PKA) which phosphorylates MLCK, opens ATP-sensitive (K_{ATP}) and large conductance calcium-sensitive (BK_{Ca}) potassium channels, resulting in hyperpolarization and relaxation. R, G-protein coupled receptor.

tetrahydrobiopterin is essential to link to two dimers of nitric oxide together, a process that is required for maximal activity (Hevel & Marletta, 1992).

There are three known subtypes of nitric oxide synthase: neuronal nitric oxide synthase (nNOS or NOS I), which was first identified in brain tissue (Bredt & Snyder, 1990) and some other tissues including peripheral nerves, lung and stomach (Schmidt *et al.*, 1992); inducible nitric oxide synthase (iNOS or NOS II) which is found in most cell types but is only expressed when induced by substances such as bacterial lipopolysaccharide (Hevel *et al.*, 1991; Stuehr *et al.*, 1991); and endothelial nitric oxide synthase (eNOS or NOS III), which is the isoform found in the vascular endothelium as well as in platelets (Radomski *et al.*, 1990). Both neuronal and endothelial nitric oxide synthase are expressed constitutively in cells and are Ca^{2+} /calmodulin sensitive and, as such, are activated by rises in the intracellular Ca^{2+} concentration; inducible nitric oxide synthase is Ca^{2+} -independent but does bind calmodulin and is activated by this co-factor, even if Ca^{2+} levels are low (Cho *et al.*, 1992).

As nitric oxide synthase requires many cofactors it can be inhibited in a variety of ways including agents which: interfere with substrate or co-factor synthesis, availability or uptake; interfere with the heme-moiety; or prevent the binding of substrate to the enzyme (for review see; Hobbs *et al.*, 1999). The most commonly used method of attenuating nitric oxide synthase activity is to prevent binding of L-arginine to the enzyme by using structural analogues (Hobbs *et al.*, 1999). The analogue of L-arginine first described as an inhibitor of nitric oxide synthase was N^G -monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987) which was used to identify many of nitric oxide's physiological actions (Moncada *et al.*, 1989). Other commonly used analogues of L-

arginine include N^G-nitro-L-arginine (L-NA) and N^G-nitro-L-arginine methyl ester (L-NAME); all are competitive, reversible inhibitors of all the nitric oxide synthase subtypes (Rees *et al.*, 1990).

1.2.2.3 Release of nitric oxide

(i) Agonist-induced release

As discussed in Section 1.2.2.1 nitric oxide (EDRF) is released from the acetylcholine-stimulated endothelium in the rabbit aorta (Furchgott & Zawadzki, 1980). Agents that increase intracellular calcium in a receptor-independent manner, such as the calcium ionophore A23187, can release nitric oxide from the endothelium. There are many other examples of agonists such as acetylcholine, substance P, bradykinin and histamine that produce nitric oxide-dependent vasodilatation (for review see; Calver *et al.*, 1993; Moncada *et al.*, 1991) by stimulating G-proteins on endothelial cells; these stimulate inositol trisphosphate production, which eventually leads to an increase in intracellular calcium by stimulating calcium influx (Schilling & Elliott, 1992), thus activating Ca²⁺/calmodulin-sensitive nitric oxide synthase.

(ii) Basal and flow-induced release

Inhibition of nitric oxide synthase induces endothelium-dependent vasoconstriction in isolated arteries from a wide range of species, including human (see below). Furthermore, cultured endothelial cells spontaneously release nitric oxide (Palmer *et al.*, 1988). It is thought that this basal release of nitric oxide reflects stimulation of nitric oxide synthase by the resting calcium concentration in the endothelial cell (Long & Stone, 1985; Miller *et al.*, 1985). This basal release of nitric oxide can also be observed as an increase in tone or pressure upon addition of an inhibitor of nitric oxide synthase

in perfused vessels (Amezcuca *et al.*, 1988), and *in vivo* (Mügge *et al.*, 1991); it is possible that *in vivo* neural release or flow-induced release may contribute to this basal release of nitric oxide. However, basal release of nitric oxide can be observed in isolated arteries where the effects of flow or neural release can be excluded (Martin *et al.*, 1986).

Flow-induced release of nitric oxide was first observed by Rubanyi *et al.* (1986) and flow-induced shear stress on endothelial cells is now known to be an important stimulus for this release. Exposure of cultured endothelial cells to shear stress leads to release of nitric oxide (Kanai *et al.*, 1995; Kuchan & Frangos, 1994) and increased expression of endothelial nitric oxide synthase (Ranjan *et al.*, 1995; Ziegler *et al.*, 1998). Shear stress causes increases in the intracellular Ca^{2+} concentration (Jen *et al.*, 2000) and opening of ion channels (Hoyer *et al.*, 1998; Olesen *et al.*, 1988); stretch-activated Ca^{2+} channels appear to be responsible for the increase of endothelial intracellular Ca^{2+} concentration produced by shear stress (Brakemeier *et al.*, 2002; Naruse & Sokabe, 1993). As well as shear stress, the heartbeat causes pulsatile distension of the vascular wall and this can release nitric oxide in the coronary circulation of dogs (Canty & Schwartz, 1994; Recchia *et al.*, 1996) and rabbits (Lamontagne *et al.*, 1992). It is thought that nitric oxide released by pulsatile stretch may be of particular importance to vasomotor control in the coronary circulation, where during systole there is virtually no flow-induced shear stress (Dube & Canty, 2001). Interestingly, flow-induced vasodilatation in the dog coronary circulation appears to be partially mediated by another factor distinct from nitric oxide (Canty & Schwartz, 1994).

(iii) neural release of nitric oxide

Neuronal nitric oxide synthase was first observed in brain tissue and this isozyme is also found in neurones supplying the cerebral vasculature (Bredt & Snyder, 1990) as well as blood vessels supplying the eye (for review see; Koss, 1999). Indeed nitric oxide is the neurotransmitter released by some non-adrenergic, non-cholinergic (NANC) nerves in the autonomic nervous system (Gillespie *et al.*, 1989; Bult *et al.*, 1990; Rand, 1992). Such nerves are now termed nitrergic nerves. With the localisation of neuronal nitric oxide synthase in some blood vessels it is unsurprising that neuronal release of nitric oxide can play a part in cardiovascular control. For example, inhibition of nitric oxide synthase can increase sympathetic nerve-induced contractions in dog mesenteric artery and attenuate parasympathetic (nitrergic) nerve-induced vasodilatations in dog cerebral arteries (Toda & Okamura, 1990); furthermore, nitric oxide is released by nitrergic nerves in the penile arteries (Burnett *et al.*, 1992), causing vasodilatation and erection by filling of the corpus cavernosum (Rajfer *et al.*, 1992).

1.2.2.4 Nitric oxide in vascular disease

Many vascular diseases such as hypertension (Linder *et al.*, 1990), hypercholesterolaemia and atherosclerosis (Shimokawa & Vanhoutte, 1989), diabetes (Timimi *et al.*, 1998; Ting *et al.*, 1996), and chronic heart failure (Ellis *et al.*, 2001; Hornig *et al.*, 1998) are associated with reduced endothelium-dependent relaxation. In addition, some ocular diseases such as glaucoma (Henry *et al.*, 1999) and diabetic retinopathy (Schmetterer *et al.*, 1997) have a vascular component and are associated with endothelial dysfunction. In human hypertension, vasodilatation to acetylcholine is reduced, as are vasoconstrictions to inhibitors of nitric oxide synthase, indicating that nitric oxide activity is reduced compared to control patients (Panza *et al.*, 1993). Indeed,

dysfunction of the nitric oxide pathway can, indirectly, lead to vascular remodelling that may facilitate the pathology of diseases such as hypertension and atherosclerosis (Taddei *et al.*, 1998a). Many of these vascular diseases are associated with oxidant stress and acute treatment with antioxidants such as vitamin C (ascorbic acid) has been shown to restore impaired nitric oxide vasodilatation in essential hypertension (Natali *et al.*, 2000; Taddei *et al.*, 1998b), chronic heart failure (Ellis *et al.*, 2001; Hornig *et al.*, 1998), atherosclerosis (Levine *et al.*, 1996), hypercholesterolaemia (Ting *et al.*, 1997) and diabetes (Timimi *et al.*, 1998; Ting *et al.*, 1996). This protective action of ascorbate may be due to its ability to scavenge the superoxide anion which destroys nitric oxide (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), but ascorbate may also help increase nitric oxide production by elevating levels of tetrahydrobiopterin, a cofactor for nitric oxide synthase (Heller *et al.*, 2001; Huang *et al.*, 2000).

Some pathological states, such as migraine, are associated with an overproduction of nitric oxide (Olesen *et al.*, 1994a). It is thought that in migraine, 5-HT stimulates nitric oxide release from endothelial cells of the cerebral vasculature. The nitric oxide then causes sensory fibres to release neurotransmitters which cause oedema, inducing pain. The ability of some nitric oxide donor drugs such as amyl nitrite to cause migraine-like headache seems to support this hypothesis (Olesen *et al.*, 1994a). Septic shock, a clinical syndrome characterised by severe hypotension and cardiovascular failure, is also associated with excess nitric oxide production. Under normal conditions production of nitric oxide by the inducible nitric oxide synthase protects against invasion by micro-organisms (Colasanti & Suzuki H., 2000). However, in septic shock bacterial endotoxins cause an over-expression of inducible nitric oxide synthase. The excess nitric oxide may be cytotoxic and causes vasodilatation leading to myocardial

depression and circulatory failure (see Forstermann *et al.*, 1994; Moncada *et al.*, 1991). Inhibitors of nitric oxide synthase are effective against septic shock in animal models (Kilbourn *et al.*, 1994) but have the disadvantage of inhibiting the constitutive isoforms as well i.e. impairing all physiological functions such as neurotransmission. The development of selective inducible nitric oxide synthase inhibitors such as 1400W (Garvey *et al.*, 1997) should offer a safer therapy for septic shock.

1.2.3 Prostacyclin

Prostacyclin (prostaglandin I₂, PGI₂) is the principal metabolite of arachidonic acid produced by cyclooxygenase in the endothelium of most blood vessels (Moncada *et al.*, 1976). Like nitric oxide, it is released from endothelial cells by a variety of stimuli, that increase intracellular Ca²⁺ such as agonist stimulation and flow-induced release, leading to vasodilatation and inhibition of platelet aggregation. Prostacyclin stimulates adenylyate cyclase on target cells leading to increases in intracellular cyclic AMP (Tateson *et al.*, 1977). In experimental settings, prostacyclin-mediated responses can be inhibited using antagonists of cyclooxygenase such as indomethacin. In most vascular preparations, prostacyclin-induced increases of smooth muscle cell cyclic AMP concentration leads to vasodilator responses by stimulation of protein kinase A, which leads to a decrease in intracellular calcium concentration and phosphorylation of MCLK. In some tissues prostacyclin can cause vasodilatation and hyperpolarization by the opening of the ATP-sensitive K⁺ channel (K_{ATP}), since these vasodilations can be inhibited by glibenclamide (Jackson *et al.*, 1993; Murphy & Brayden, 1995). In addition, in some tissues prostacyclin-induced vasodilatation can involve the opening of large-conductance, calcium-sensitive potassium channels (BK_{Ca}) (Schubert *et al.*, 1997) resulting in hyperpolarization of the smooth muscle cells leading to a decrease in the

intracellular calcium concentration, causing relaxation. An overview of prostacyclin-induced vasodilatation can be seen in Figure 1.6.

1.2.4 Endothelium-derived hyperpolarizing factor

In some vascular preparations a significant vasodilator response can still be observed after inhibition of both the nitric oxide and prostacyclin pathways. This vasodilator pathway is associated with a hyperpolarization of the underlying smooth muscle and is believed to be mediated by an agent termed endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.*, 1988; Feletou M & Vanhoutte P.M, 1988). In general it would appear that nitric oxide is the predominant endogenous vasodilator in larger vessels and EDHF assumes more importance in smaller arteries (Adeagbo & Triggle, 1993; Garland *et al.*, 1995; Nagao *et al.*, 1992); however, EDHF-mediated vasodilatations can be observed in many large conduit arteries such as the porcine (Fisslthaler *et al.*, 1999; Fleming *et al.*, 1999) and bovine (Campbell *et al.*, 1996; Drummond & Cocks, 1996) coronary arteries. Like nitric oxide, EDHF can be released by flow and, in the coronary circulation of humans and pigs, EDHF appears to be released by both shear stress (Dube & Canty, 2001; Miura *et al.*, 2001) and pulsatile stretch (Popp *et al.*, 1998).

1.2.4.1 Mechanism of action of endothelium-derived hyperpolarizing factor

It is generally accepted that EDHF's relaxant mechanism involves the hyperpolarization of vascular smooth muscle cells; this hyperpolarization of the smooth muscle cell leads to a decrease in the intracellular Ca^{2+} concentration by reducing the open probability of L-type voltage-dependent calcium channels, leading to vasodilatation (Feletou & Vanhoutte, 1999).

The initial step in the EDHF response appears to be a rise in the intracellular Ca^{2+} concentration of endothelial cells (Chen & Suzuki, 1990). Indeed, agonists that evoke EDHF-mediated responses by activating endothelial G-protein-coupled receptors cause a rise in the intracellular Ca^{2+} concentration (Busse *et al.*, 2002). Furthermore, EDHF-mediated responses can be elicited by agents which can increase the endothelial intracellular Ca^{2+} concentration, such as the calcium ionophore A23187 (Hutcheson *et al.*, 1999; Plane *et al.*, 1995) or the inhibitors of Ca^{2+} -ATPase, thapsigargin and cyclopiazonic acid (Fukao *et al.*, 1995; Illiano *et al.*, 1992).

EDHF-mediated vasodilatation involves increases in K^+ conductance as it is sensitive to depolarising concentrations of K^+ (above 25 mM; Adeagbo & Triggle, 1993) and inhibitors of certain K^+ channels (see below). Furthermore, EDHF-mediated responses are associated with an increase in rubidium efflux (Chen *et al.*, 1988; Taylor *et al.*, 1988) and decreases in membrane resistance, suggesting that they are due to the opening of a K^+ channel, not the closing of a chloride channel, nor the closing of some other cation conductance (Feletou & Vanhoutte, 1999). An overview of EDHF-mediated vasodilatation can be found in Figure 1.7.

1.2.4.2 Characterisation and location of the K^+ channels involved in the EDHF response

Endothelium-dependent hyperpolarizations resistant to blockade of NOS and cyclooxygenase are not sensitive to the blocker of ATP-sensitive K channels (K_{ATP}), glibenclamide (Busse *et al.*, 2002). In contrast, non-selective inhibition of Ca^{2+} -sensitive potassium channels (K_{Ca}) with tetraethylammonium inhibits EDHF-mediated responses

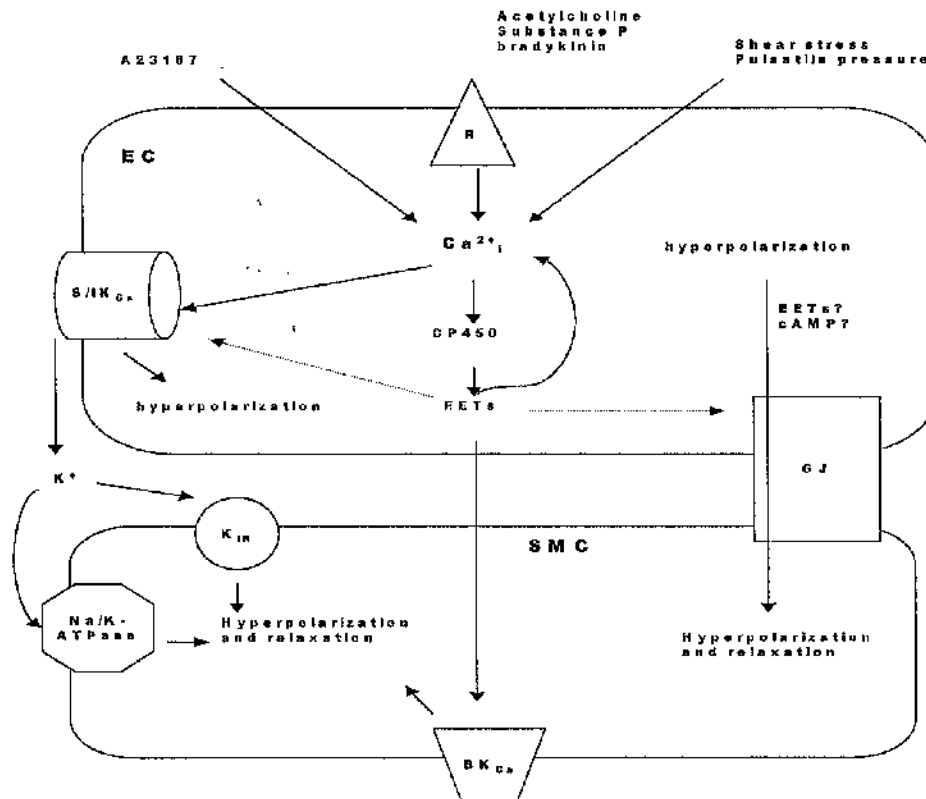


Figure 1.7 Schematic diagram showing the current hypotheses of how EDHF may function (see text for details). Rises in intracellular $[Ca^{2+}]$ (Ca^{2+}_i) may cause endothelium-dependent vasodilatation in several ways. (i) Activation of small and intermediate calcium-sensitive K channels (S/IK Ca_v), will release K^+ into the intracellular space, thus stimulating Na/K-ATPase or inward-rectifier K (K_{IR}) channels on smooth muscle cells (SMC), causing hyperpolarization and relaxation. (ii) Stimulation of cytochrome P450 (CP450) in endothelial cells (EC) may produce EETs, which could either directly stimulate large conductance calcium-sensitive K channels (BK Ca_v) on SMC or modulate myo-endothelial gap junctions (GJ), endothelial K Ca_v channels, or Ca^{2+} release from endothelial stores. Hyperpolarization may spread electrotonically from the EC to SMC *via* GJs; alternatively a diffusible substance may pass through GJs to hyperpolarize SMC.

(Chen & Cheung, 1997; Hioning *et al.*, 2000). Furthermore, blocking K_{Ca} channels with specific toxins causes blockade of EDHF-mediated responses; the characteristic blockade of EDHF is by the combination of apamin, an inhibitor of small-conductance K_{Ca} (SK_{Ca}) channels (Castle, 1999) and charybdotoxin a non-selective inhibitor of large and intermediate conductance K_{Ca} channels (Garcia *et al.*, 1991)(BK_{Ca} and IK_{Ca} , respectively) and voltage gated K^+ channels (K_v) (Corriu *et al.*, 1996; Garland & Plane, 1996; Zygmunt & Hogestatt, 1996). In some preparations either apamin (bovine coronary artery) (Drummond *et al.*, 2000) or charybdotoxin (human coronary artery) (Miura *et al.*, 1999) alone can inhibit EDHF-mediated responses. In preparations where the combination of charybdotoxin and apamin is required to block EDHF-mediated responses, the selective BK_{Ca} inhibitor, iberiotoxin (Garcia *et al.*, 1991), cannot substitute for charybdotoxin (Zygmunt & Hogestatt, 1996); thus, EDHF-mediated responses involve the opening of both SK_{Ca} and IK_{Ca} but not BK_{Ca} channels.

There are no reports of K_{Ca} channels on vascular smooth muscle cells that are sensitive to charybdotoxin but not iberiotoxin. In other words, it is unlikely there are IK_{Ca} channels on vascular smooth muscle cells. In contrast, both SK_{Ca} and IK_{Ca} are present in endothelial cells (Burnham *et al.*, 2002; Cai *et al.*, 1998). Indeed, it has been observed that endothelial K^+ channels are activated by rises in intracellular Ca^{2+} concentration (Busse *et al.*, 1988). Furthermore, endothelial cell hyperpolarization elicited by either acetylcholine, in rat hepatic arteries (Edwards *et al.*, 1998), or bradykinin, in the porcine coronary artery, (Edwards *et al.*, 2000), is inhibited by the combination of charybdotoxin and apamin. In addition, the putative opener of IK_{Ca} channels, 1-EBIO (Adeagbo, 1999) can induce hyperpolarization of endothelial cells associated with an

endothelium-dependent hyperpolarization of smooth muscle cells (Coleman *et al.*, 2001; Edwards *et al.*, 1999a). However, care should be taken with interpreting results with 1-EBIO as Walker and colleagues (Walker *et al.*, 2001) found that it could induce relaxation of smooth muscle at concentrations below those required to produce endothelium-dependent hyperpolarization. Nevertheless, the majority of evidence suggests that when EDHF responses are blocked by the combination of tetrodotoxin and apamin, this occurs through inhibition of SK_{Ca} and IK_{Ca} located on the endothelium, rather than on smooth muscle cells.

1.2.4.3 The nature of endothelium-derived hyperpolarizing factor

There have been many different hypotheses on the chemical nature of EDHF. Suggestions have included the endogenous cannabinoid, anandamide (Randall *et al.*, 1996), epoxyeicosatrienoic acids (Campbell *et al.*, 1996), potassium ions (Edwards *et al.*, 1998), nitric oxide (Cohen *et al.*, 1997) and hydrogen peroxide (Matoba *et al.*, 2000); for a recent review see (Feletou & Vanhoutte, 2000)). Indeed, none of these candidates can be ruled out as all can be synthesised by the endothelium and can cause hyperpolarization of the underlying smooth muscle. However, the vast majority of evidence currently supports three hypotheses: (i) hyperpolarization is transmitted directly to the smooth muscle from the endothelial cell via gap junctions; (ii) the increase in endothelial intracellular Ca²⁺ activates cytochrome P450 and the resultant metabolites are essential for EDHF-mediated responses; (iii) K⁺ is released from endothelial cells by the opening of K_{Ca} channels and the elevated concentration of K⁺ in the extracellular space hyperpolarizes smooth muscle by activating the inward rectifier (K_{IR}) or Na/K-ATPase on the smooth muscle cells. Of course, it is possible that some combination of these mechanisms may account for the EDHF-response in any particular

tissue, and the relative importance of each mechanism may differ among different vascular beds.

(i) Gap Junctions

Hyperpolarization of the endothelium causes hyperpolarization of vascular smooth muscle with the same time course (Beny, 1990); perhaps by electrotonic spread of hyperpolarization through myo-endothelial gap junctions. Indeed, myo-endothelial gap junctions could provide the low-resistance electrical pathway that would be necessary to couple the endothelial cells to smooth muscle cells (Christ *et al.*, 1996). Furthermore, it has been demonstrated that the number of myo-endothelial gap junctions increases as vessel diameter decreases in the mesenteric arterial bed of the rat (Sandow & Hill, 2000); this correlates well with the observation that the contribution of EDHF to vasomotor control increases as vessel diameter decreases. It has been demonstrated that EDHF-mediated responses can be inhibited using the specific inhibitors of gap junctions, the GAP peptides (e.g. GAP 27) (Chaytor *et al.*, 1998; Chaytor *et al.*, 2001). However, the ability of GAP peptides to inhibit gap-junctions is only inferred from their ability to prevent dye coupling in cultured cells (Chaytor *et al.*, 2001) and it is only an assumption that the myo-endothelial gap junctions are the target for GAP peptides; it is also possible that they could affect endothelial-endothelial or smooth muscle-smooth muscle gap junctions. Other agents such as 18 α -glycyrrhetic acid and carbonexolone have been used to investigate the role of gap junctions in EDHF-mediated response. However these agents are notoriously non-specific and can inhibit the Na/K-ATPase, therefore, care should be taken in interpreting results obtained with such agents.

Another possibility is that a diffusible substance (e.g. EDHF), rather than electrical current, could pass through myo-endothelial gap junctions. Such a mediator would have

to be small in size and hydrophilic in order to pass through the aqueous pore of the gap junction and would need to be generated in large quantities over a sustained period in order to overcome extensive dilution when transferred to the underlying smooth muscle (Busse *et al.*, 2002). Cyclic AMP appears to serve as a diffusible endothelium-dependent hyperpolarizing factor (Chaytor *et al.*, 2001). However, it seems unlikely that transfer of cyclic AMP through myo-endothelial gap junctions underpins the EDHF response, since relaxing smooth muscle with prostacyclin elevates cyclic AMP resulting in the opening of K_{ATP} channels. However, EDHF-mediated responses are unaffected by blockade of K_{ATP} channels therefore cyclic AMP is more likely to be involved in regulation of gap junctions than actually functioning as EDHF (Busse *et al.*, 2002).

(ii) Cyclooxygenase P450 metabolites

It has been suggested that epoxyeicosatrienoic acids (EETs), short-lived metabolites of arachidonic acid produced by cytochrome P450, could account for EDHF-mediated responses in coronary arteries from a wide range of species including cattle (Bauersachs *et al.*, 1994; Campbell *et al.*, 1996) and pigs (Bauersachs *et al.*, 1994; Popp *et al.*, 1996). Unfortunately, most of the blockers of cytochrome P450 used in these experiments are notoriously non-specific. Nevertheless, EDHF-mediated responses were inhibited in experiments with newer, more selective blockers of cytochrome P450 such as sulfaphenazole, a selective inhibitor of cytochrome P450 2C9 (Fisslthaler *et al.*, 2000), or with anti-sense oligonucleotides (Fisslthaler *et al.*, 1999). It has also been demonstrated that EETs can relax and hyperpolarize coronary arteries by increasing open probability of K_{Ca} channels (Campbell *et al.*, 1996; Fisslthaler *et al.*, 1999). Taken together this evidence would suggest that EETs are a strong candidate for EDHF, at least in coronary arteries (Busse *et al.*, 2002).

There is no doubt that EETs are synthesised by endothelial cells (Campbell *et al.*, 1996), however, there is little evidence that they can be released in sufficient amounts to cause relaxation and hyperpolarization of smooth muscle cells. Furthermore, the K_{Ca} channels activated by EETs are BK_{Ca} (Zhang *et al.*, 2001) rather than SK_{Ca} or IK_{Ca} , again indicating that diffusible EETs are unlikely to account for most EDHF-mediated responses. EETs could, however, act within the endothelium to regulate intracellular Ca^{2+} , possibly by regulating intracellular Ca^{2+} stores (Hoebel *et al.*, 1997), by increasing endothelial K_{Ca} channel sensitivity to Ca^{2+} (Baron *et al.*, 1997), or even modulating gap junction permeability (Popp *et al.*, 2002).

(iii) K^+ as endothelium-derived hyperpolarizing factor

Small increases in extracellular potassium concentration can produce vasodilatation and increases in local blood flow that is physiologically significant. Indeed K^+ loss from contracting skeletal muscles increases blood flow to the active muscle (Juel *et al.*, 2000; Skinner & Powell, 1967). Similarly, efflux of K^+ from active areas of the CNS produces localised vasodilatation, presumably helping to supply nutrients to these areas (Kuschinsky & Wahl, 1978; McCarron & Halpern, 1990). It has been proposed that opening of endothelial cell K_{Ca} currents leads to an accumulation of K^+ in the extracellular space between endothelial and smooth muscle cells, resulting in a hyperpolarization and relaxation of the smooth muscle by activating inward rectifier K^+ (K_{IR}) and Na/K-ATPase (Edwards *et al.*, 1998; Nelson & Quayle, 1995). Indeed, EDHF-mediated responses can be attenuated in the rat hepatic artery by using barium (30 μ M) to inhibit K_{IR} and ouabain to inhibit the Na/K-ATPase (Edwards *et al.*, 1998). Furthermore, in the rat mesenteric and hepatic arteries (Edwards *et al.*, 1998), mouse

mesenteric arteries (Ding *et al.*, 2000), and porcine coronary arteries (Beny & Schaad, 2000), exogenously applied K^+ (<25 mM) can mimic the effect of EDHF; i.e. it causes hyperpolarization (and relaxation) of the smooth muscle, indicating that EDHF may simply be K^+ released from the endothelium.

The idea that K^+ derived from the endothelium is EDHF has been challenged, and in many vascular preparations where EDHF-mediated responses can be observed, exogenously applied K^+ does not evoke consistent smooth muscle hyperpolarization or relaxation (Doughty *et al.*, 2000; Edwards *et al.*, 1999b; Harris *et al.*, 2000; Lacy *et al.*, 2000). However, a simple explanation may account for such variability: In some tissues the researchers were studying vascular tone and therefore used a vasoconstrictor agent. In such experiments where tension recording and hyperpolarization were measured simultaneously, it was demonstrated that the ability of K^+ to mimic EDHF diminished as agonist-induced tone was increased (Dora & Garland, 2001; Richards *et al.*, 2001). Therefore, it is possible that when vasoconstrictor tone is high, the resultant depolarisation and increase in intracellular Ca^{2+} in smooth muscle cells causes activation of K_{Ca} , forming a K^+ “cloud” in the intercellular space already maximally stimulating their K_R or Na/K-ATPase. Thus, additional K^+ released from the endothelium following activation of SK_{Ca} and IK_{Ca} will not further stimulate these processes.

1.2.4.4 Endothelium-derived hyperpolarizing factor in vascular disease

Diseases that cause endothelial dysfunction are likely to affect EDHF-induced responses, just as they affect nitric oxide-mediated responses. However there is relatively little information on the role of EDHF in pathophysiology. There are reports

of a reduction of endothelium-dependent vasodilatation that is nitric oxide- and prostacyclin-independent (Fukao *et al.*, 1997) (Makino *et al.*, 2000) in experimentally-induced diabetes, which may be indicative of a reduced role of EDHF in this condition. However, it has been reported that diabetes can potentiate EDHF-mediated relaxation in the rabbit renal artery (Alabadi *et al.*, 2001). This may reflect a compensatory mechanism for the apparent loss of nitric oxide-mediated vasodilatation found in this study. In isolated coronary arteries from patients with atherosclerosis, endothelium-dependent responses are unaffected despite a reduction in the contribution of nitric oxide (Garland *et al.*, 1995); the EDHF component of vasodilatation appears to compensate for loss of nitric oxide in these arteries (Selemdis & Cocks, 2002).

1.3 Vasodilator mechanisms in the eye

The anatomy of the vascular system of the bovine eye has already been discussed in Section 1.1.2. This section will describe the current understanding of the contribution of nitric oxide, prostacyclin and EDHF to vasomotor control in the eye.

1.3.1 Nitric oxide

There is a wealth of evidence to suggest that nitric oxide is an important regulator of vascular tone in the eye (for review see, Koss, 1999). Furthermore, nitric oxide synthase has been shown to be located in many regions of the eye (Koss, 1999), including the nerve supply to the arterial circle of the iris (Tamm *et al.*, 1995). There is also heavy immunostaining for nitric oxide synthase throughout the vascular endothelium of the choroid in a wide range of species (Koss, 1999).

1.3.1.1 Basal release

There is compelling evidence that there is a basal release of nitric oxide in the blood vessels of the eye, since inhibition of nitric oxide synthase causes a reduction in the basal blood flow in a wide range of species, as measured using radiolabelled microspheres (Hardy *et al.*, 1996; Nilsson, 1996; Seligsohn & Anders, 2000) or laser Doppler flowmetry (Koss, 1998; Luksch *et al.*, 2000; Zagvazdin *et al.*, 1996). It remains to be determined whether this tonic vasodilator influence of nitric oxide on the ocular vasculature *in vivo* is derived from the endothelium as a result of basal release, release stimulated by agonists (Benedito *et al.*, 1991a; Gidday & Zhu, 1995), or flow (Rubanyi & Vanhoutte, 1986), or release from perivascular nitrenergic nerves (Nilsson, 1996; Toda *et al.*, 1998; Zagvazdin *et al.*, 1996). Nevertheless, in studies using the porcine isolated perfused eye, where neural influences can be excluded (Meyer *et al.*, 1993), and in isolated segments of ophthalmic, ciliary, retinal and irideal arteries from a variety of species including pig, cattle and rat (Benedito *et al.*, 1991a) (Haeffliger *et al.*, 1993) (Hill & Gould, 1995; Su *et al.*, 1994; Yao *et al.*, 1991), where the effects of both flow and nerves can be excluded, augmentation of vasoconstrictor tone by inhibitors of nitric oxide synthase provides evidence of basal release of nitric oxide from the endothelium in the ocular circulation.

1.3.1.2 Agonist-stimulated endothelial release

There are numerous reports of agonist-induced, endothelium-dependent vasodilator responses which are inhibited by antagonists of nitric oxide synthase in ocular arteries in a wide range of species (Koss, 1999). For example, *in vivo* vasodilatations to acetylcholine in new born pigs (Gidday & Zhu, 1995), or to substance P in dogs (Kitamura *et al.*, 1993), are sensitive to nitric oxide synthase inhibitors. *In vitro*, using the porcine isolated perfused eye preparation (cannulated via the ophthalmic artery),

vasodilatations induced by bradykinin were inhibited by L-NAME. Furthermore, in isolated ocular vessels inhibition of nitric oxide synthase can inhibit vasodilatation to acetylcholine in cattle (Benedito *et al.*, 1991a, b), pigs (Gidday & Zhu, 1995; Yao *et al.*, 1991), and guinea pigs (Tamai *et al.*, 1999a); and to bradykinin in pigs (Haeffliger *et al.*, 1993; Yao *et al.*, 1991; Zhu *et al.*, 1997).

1.3.1.3 Neural release

It is highly likely that neural release of nitric oxide modulates vasomotor tone in the eye. There is evidence showing localisation of nitric oxide synthase with the parasympathetic nerves that innervate the ocular blood vessels (Cuthbertson *et al.*, 1997; Tamm *et al.*, 1995; Toda *et al.*, 1994; Toda *et al.*, 1996). Evidence from neuroanatomical studies suggests that these nerves arise from the pterygopalatine ganglion *via* the facial nerve in mammals (Nilsson, 1996) and from the ciliary ganglion in birds (Zagvazdin *et al.*, 1996). Furthermore, parasympathetically mediated vasodilatation in the eye, *in vivo*, is inhibited by nitric oxide synthase inhibitors in rabbits (Nilsson, 1996) and pigeons (Zagvazdin *et al.*, 1996). A role for neural release of nitric oxide in ocular vasomotor control is supported by experiments with isolated ocular arteries stimulated by transmural electrical stimulation; in these experiments, parasympathetic neural vasodilatations were attenuated by inhibitors of nitric oxide synthase in ciliary arteries of monkeys (Toda *et al.*, 1998), cattle (Wiencke *et al.*, 1994), dogs (Toda *et al.*, 1999), and pigs (Su *et al.*, 1994; Toda *et al.*, 1997).

1.3.2 Prostacyclin and endothelium-derived hyperpolarizing factor

In contrast to the overwhelming evidence supporting the involvement of nitric oxide in ocular vasomotor control, there is little evidence of endogenous prostacyclin being of

much importance. Exogenously added prostacyclin does, however, cause vasodilatation in bovine isolated small retinal arteries (Benedito *et al.*, 1991), in the porcine isolated perfused eye (Meyer *et al.*, 1993) and in the ocular circulation of newborn pigs (Hardy *et al.*, 1996).

Prior to this study there have also been very few reports of EDHF-mediated vasodilatation being involved in ocular vasomotor control, indeed in the examples listed below the authors never suggested that EDHF mediated the vasodilator responses observed. In isolated porcine ciliary artery the endothelium-dependent vasodilatation is mediated primarily by nitric oxide (Zhu *et al.*, 1997), but after inhibition of nitric oxide synthase, a residual vasodilator response insensitive to glibenclamide (an inhibitor of K_{ATP} channels) but abolished by TEA (an inhibitor of K_{Ca}) was observed, which may have been mediated by EDHF. In addition, in guinea pig choroidal arterioles, the majority of the vasodilator response is resistant to inhibition of nitric oxide synthase and cyclo-oxygenase (Tamai *et al.*, 1999b). This vasodilator response was attenuated by TEA, apamin or charybdotoxin; furthermore, the combination of apamin and charybdotoxin virtually abolished this vasodilatation, strongly indicating that it was mediated by EDHF.

1.4 Aim

The aim of the study was to characterise endothelium-dependent vasodilator responses in the ciliary vascular bed of the bovine eye. As will be seen, these were found to be mediated almost entirely by an EDHF-like substance.

In the course of this investigation, a chance observation revealed that endothelium-dependent vasodilatation induced by acetylcholine or bradykinin in the ciliary vascular bed of the bovine eye was blocked by ascorbate. This observation was intriguing since ascorbate is known to potentiate the actions of nitric oxide by protecting it under conditions of oxidant stress (Dudgeon *et al.*, 1998; Fontana *et al.*, 1999). In the eye, ascorbate delivered in the plasma is greatly concentrated by the ciliary epithelium in its production of aqueous humour. As the concentration of ascorbate in the aqueous humour is so high (~ 1mM), studies of aqueous humour dynamics using the bovine isolated perfused eye preparation have routinely included ascorbate (50 μ M) in the perfusion medium (Shahidullah & Wilson, 1999; Wilson *et al.*, 1993). This was to ensure the aqueous humour formed was similar in composition to that formed *in vivo*.

The remainder of the study was thus devoted to characterising the ability of ascorbate to block EDHF in the ciliary vascular bed of the bovine eye and in several other preparations where EDHF contributes to vasodilatation.

Chapter 2

Methods



2.1 Bovine eye

2.1.1 Preparation of the bovine isolated arterially perfused eye

The ciliary vascular bed of the bovine eye was perfused using a modification of the constant flow method first described by Wilson *et al.* (1993). Bovine eyes were obtained from a local abattoir and transported to the laboratory at ambient temperature; previous work had shown that transport on ice caused the fatty tissue around the orbit to solidify, making cannulation difficult (Wilson *et al.*, 1993). Also it is very likely that cooling would slow the return of the eye's core temperature to 37°C during experiments, which was considered undesirable. In the laboratory excess tissue was removed from the globe of the eye, with care to avoid damaging the blood vessels running over the surface of the eye. However, a few mm of extraocular muscle was left attached to the globe to aid location of vortex veins. A long posterior ciliary artery was then selected, carefully cleaned, then cannulated at a point proximal to the pigmented region where it enters the sclera and distal to where it leaves the ophthalmic artery. The cannulae used were hand-made from polythene tubing (bore 2.5 mm, wall thickness 1 mm). The eye was then placed in a heating jacket, and covered with tissue and foil for insulation. The eye cannulated via the ciliary artery was then perfused at 37°C with Krebs solution containing mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.2; MgSO₄ 1.2; NaHCO₃, 25; glucose, 11.5; and gassed with O₂ containing 5% CO₂, to adjust the pH to 7.4 (Figure 2.1).

Perfusion of the eye was normally begun within 90 min of slaughter; successful perfusion was indicated by flow of a small amount of blood from the cut ends of the vortex veins. The flow was induced by peristaltic pumps (Watson-Marlow, model 502S; Figure 2.1) and was commenced at a rate of ~0.2-0.5 ml min⁻¹ and gradually

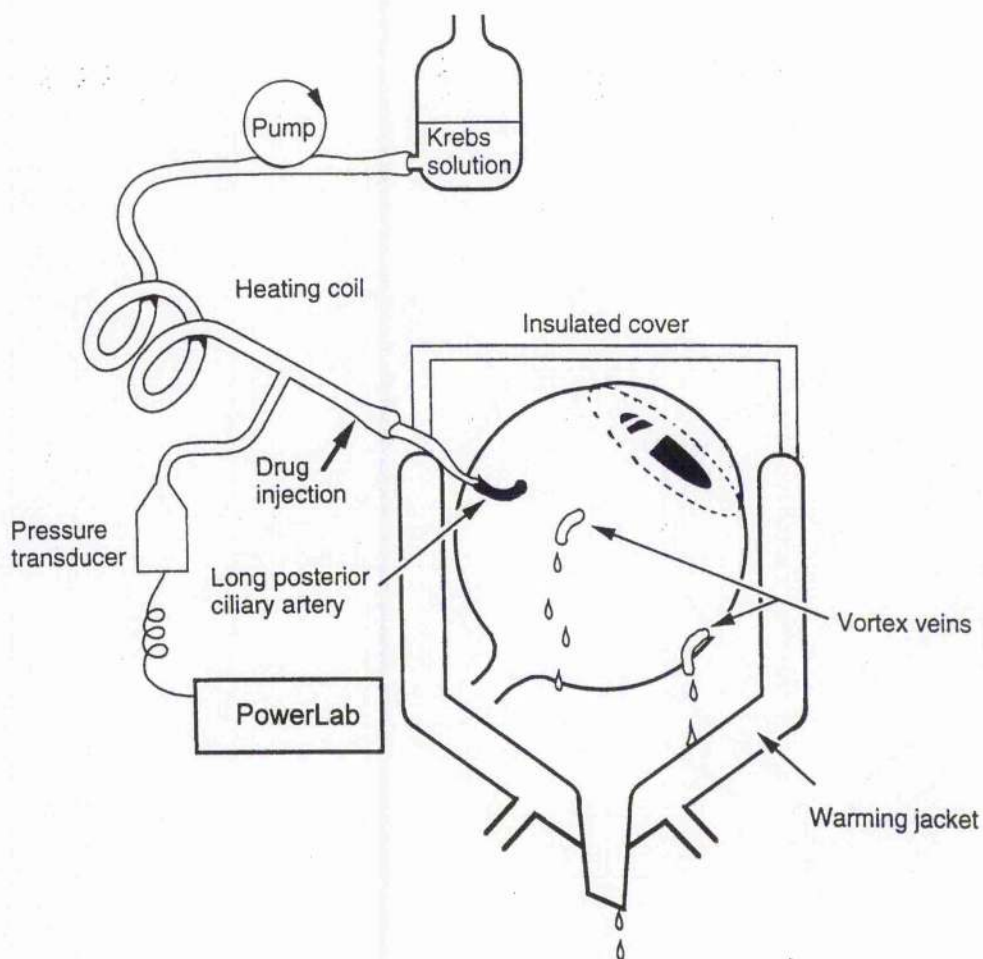


Figure 2.1 The bovine isolated perfused eye preparation and apparatus showing perfusion of the ciliary vascular bed through one long posterior ciliary artery. The perfusion pressure was measured by a pressure transducer *via* a sidearm located immediately proximal to the inflow cannula and was displayed and recorded using a PowerLab data acquisition system.

increased in 5-10 increments to a final flow rate of 2.5 ml min^{-1} over a 50 min period. The flow was increased in this gradual manner to allow the eye to warm up and to prevent damaging the microvasculature of the eye. After the final flow rate was achieved, eyes were perfused for an equilibration period of at least 30 min before starting the experimental protocols. Perfusion pressure was measured by pressure transducers (Gould Statham, P32ID) *via* a side arm located immediately proximal to the inflow cannula and displayed and recorded using a computer system (PowerLab/800, ADInstruments, Hastings, UK; figure 2.1). Only eyes that had a basal perfusion pressure of 20-60 mmHg were used for further study.

2.1.2 Experimental Protocols with the bovine isolated arterially perfused eye

2.1.2.1 Basal perfusion pressure

After the equilibration period, drugs were added either to the Krebs reservoir for continuous infusion, or as bolus doses injected through rubber tubing immediately proximal to the infusion cannula (figure 2.1). Experiments were conducted to assess the effects of a range of drugs on the basal perfusion pressure. These drugs were: the nitric oxide synthase inhibitor, L-NAME (100 μM ; Rees *et al.*, 1990); the soluble guanylate cyclase inhibitor, ODC (10 μM ; Garthwaite *et al.*, 1995) both to investigate the role of nitric oxide; and the non-selective phosphodiesterase inhibitor, papaverine (150 μM ; Martin *et al.*, 1986). In these experiments the drugs were infused for at least 20 min and any response allowed to stabilise before measurements of their effects on perfusion pressure were recorded.

2.1.2.2 Vasoconstrictor screen and general experimental procedure for experiments involving vasodilator responses

Experiments were conducted to select a suitable vasoconstrictor agent. These involved constructing cumulative concentration-response curves to a range of commonly used vasoconstrictor agonists. These drugs were: the endogenous adrenoceptor agonist, noradrenaline; the synthetic α_1 -adrenoceptor agonist, phenylephrine; the thromboxane A_2 -mimetic, U46619; the prostaglandin, prostaglandin $F_{2\alpha}$; and the endogenous neurotransmitter agonist, 5-hydroxytryptamine (5-HT). In these experiments vasoconstrictor responses to each concentration of agonist, infused continuously, were allowed to stabilise before a higher concentration was added. Noradrenaline and 5-HT are unstable in aqueous solution so ascorbic acid (50 μ M) and EDTA (10 μ M) were added to the perfusate to prevent them from oxidising.

The effect of the NOS synthase inhibitor, L-NAME was assessed on perfusion pressure elicited by 5-HT (300 nM), noradrenaline (100 μ M) or U46619 (100 nM). These concentrations of the vasoconstrictor agonists were chosen so that the perfusion pressure was similar in each experiment. In these experiments the increase in perfusion pressure produced by the vasoconstrictors was allowed to stabilise before infusion of L-NAME (100 μ M) for 20 min or until tone re-stabilised.

Vasodilatation to bolus doses of acetylcholine was also assessed on perfusion pressure induced by sub-maximal concentrations of 5-HT, noradrenaline or U46619. In these experiments after vasoconstrictor tone stabilised, acetylcholine-induced vasodilatations were elicited by injecting 10 μ l volumes of the appropriate concentration with a Hamilton micro-syringe.

In all subsequent experiments where vasodilatations were elicited, continuous infusion of U46619 at a concentration of 50-500 nM was chosen to achieve a sub-maximal perfusion pressure (~130 mmHg) that was suitable for conducting experiments with vasodilators. In some experiments full dose-response curves to: acetylcholine (1 pmol-100 nmol); bradykinin (0.1 pmol-10 nmol); the K_{ATP} channel opener, levcromakalim (0.1-30 nmol; Katnik & Adams, 1997); the putative openers of IK_{Ca} channels, 1-EBIO (1 nmol – 1 μ mol; Adeagbo, 1999; Edwards *et al.*, 1999a) and DCEBIO (0.1 nmol – 1 μ mol; Singh *et al.*, 2001); the opener of BK_{Ca} channels, NS1619 (1 – 100 nmol; Olesen *et al.*, 1994b); and the endogenous cannabinoid, anandamide (1 – 100 nmol; Randall *et al.*, 1996), were constructed. In other experiments only a single dose of acetylcholine (10 nmol), bradykinin (10 pmol) or the nitric oxide donor, glyceryl trinitrate (GTN, 10 nmol) was used. The endothelial dependence of vasodilator responses was assessed by infusing the detergent CHAPS (0.3 %, 2 min), to selectively damage the endothelial cell layer (Randall & Hiley, 1988).

2.1.2.3 Characterisation of vasodilator responses

The effects of a number of blocking drugs were examined in order to characterise the nature of vasodilator responses to acetylcholine and bradykinin. These drugs were: the nitric oxide synthase inhibitor, L-NAME (100 μ M); the inhibitor of soluble guanylate cyclase, ODQ (10 μ M); the cyclooxygenase inhibitor, flurbiprofen (30 μ M); the non-selective K_{Ca} channel blocker, TEA (10 mM; Cook & Quast, 1990); the K_{ATP} channel blocker, glibenclamide (10 μ M; Katnik & Adams, 1997); the non-selective intermediate and large K_{Ca} (IK_{Ca} / BK_{Ca}) and K_v channel blocker, charybdotoxin (10 nM; Smith *et al.*, 1986); the selective large conductance K_{Ca} (BK^+_{Ca}) channel blocker, iberiotoxin (100 nM; Candia *et al.*, 1992); the selective small conductance K_{Ca} (SK_{Ca}) channel blocker,

apamin (100 nM; Robertson & Steinberg, 1990); the K_{IR} channel blocker, Ba^{2+} (30 μ M; Edwards & Hirst, 1988); the Na/K ATPase inhibitor, ouabain (10 μ M; Feletou & Vanhoutte, 1988); and non-selective blockade of K^+ channels by a high K^+ (30 mM; Adeagbo & Triggle, 1993) concentration in the Krebs solution. In experiments with 30 mM K^+ , a proportionate reduction in the NaCl concentration was made in order to maintain isotonicity. For each experiment the blocking drug was infused for at least 20 min before the effects on the vasodilator response were tested. In some experiments the blocking drugs themselves (L-NAME, ODQ, TEA, high $[K^+]$, charybdotoxin and iberiotoxin) affected the U46619-induced perfusion pressure, these effects are described in the first Results section (Chapter 3). Therefore, where necessary, the concentration of U46619 was adjusted so that the perfusion pressure was similar to that seen in control experiments.

2.1.2.4 Characterisation of effects of ascorbate on EDHF-mediated vasodilator responses

As will be shown in Chapter 3 the vasodilator responses to both acetylcholine and bradykinin in the bovine eye are mediated entirely by an EDHF-like substance and do not involve a contribution of a nitric oxide or cyclooxygenase product. Therefore, inhibitors of nitric oxide synthase or cyclo-oxygenase were not used to study the EDHF-like responses in this preparation.

Experiments were conducted to test the effects of ascorbate on vasodilator responses in the bovine eye. In such experiments ascorbate was included in the perfusion medium. As will be seen in chapter 4, acetylcholine elicited only vasodilator responses when bovine eyes were perfused with normal Krebs solution. In contrast, when eyes were

perfused from the outset of the experiment with Krebs solution containing ascorbate (50 μ M, > 120 min), acetylcholine produced vasoconstrictor responses, immediately followed by a residual vasodilator response.

Experiments were conducted to assess the time course of the ability of ascorbate to reverse acetylcholine-induced vasodilatation to vasoconstriction. In control experiments, acetylcholine (10 nmol)-induced vasodilator responses were elicited every 15 min during a 120 min study period in eyes perfused from the outset with Krebs solution in order to assess their reproducibility. When effects of ascorbate were assessed, control acetylcholine-induced vasodilator responses were allowed to stabilise before inclusion of ascorbate in the perfusion medium. The effects of ascorbate on acetylcholine-induced responses, elicited every 15 min, were then assessed during the ensuing 120 min. During this period, the vasodilator response declined and a vasoconstrictor response developed, often resulting in a biphasic response. Consequently, vasodilator and vasoconstrictor components were measured separately and graphed at each of the 15 min time points. Similar experiments were conducted to determine if ascorbate had any effect on vasodilator responses to bradykinin (10 pmol) in the bovine eye.

The ability of other drugs to mimic the effects of ascorbate on acetylcholine-induced responses were also assessed in similar time course experiments. These drugs were: the redox-inactive analogue, dehydroascorbate (50 μ M;), the thiol reducing agents, glutathione (1 mM; Kosower & Kosower, 1978), N-acetyl-L-cysteine (1mM; Weinander *et al.*, 1994) and dithiothreitol (100 μ M; Faulstich & Heintz, 1995), and the antioxidant enzymes, superoxide dismutase (250 units ml⁻¹) and catalase (1250 units ml⁻¹)

¹). Experiments were also conducted to assess if the blocking effects of ascorbate could be reversed by washing. In such experiments, from the outset, eyes were perfused with Krebs containing ascorbate (50 μ M, 120 min). When reproducible acetylcholine (10 nmol)-induced control vasoconstrictor responses had been obtained, perfusion then switched to ascorbate-free Krebs. Changes in acetylcholine-induced responses were then assessed for the ensuing 120 min.

Experiments were conducted to assess the concentration-dependence of the ability of ascorbate to reverse acetylcholine-induced vasodilatation to vasoconstriction. In control experiments full dose-response curves to acetylcholine were constructed in eyes perfused with normal Krebs solution. Full dose-response curves were also constructed in eyes that had been perfused from the outset (>120 min) of the experiment with Krebs containing 10, 50 or 150 μ M of ascorbate. These dose-response curves were compared to those obtained in control eyes. The vasodilator and vasoconstrictor responses to acetylcholine were plotted and compared separately.

Experiments were conducted to assess the nature of the vasoconstrictor response. In these experiments eyes were perfused from the outset with Krebs solution containing ascorbate (50 μ M, >120 min). When reproducible acetylcholine (10 nmol)-induced vasoconstrictor responses had been obtained, the effect of the cyclooxygenase inhibitor, flurbiprofen (30 μ M), and the non-selective muscarinic antagonist, atropine (100 nM), were assessed.

2.1.2.5 Selectivity of effect of ascorbate on EDHF-mediated vasodilatation

Experiments were conducted to investigate the selectivity of the blockade of EDHF-mediated vasodilatation by ascorbate. In these experiments a control dose-response curve was constructed to the ATP-sensitive potassium channel (K_{ATP}) opener, levcromakalim (0.1-30 nmol). This was compared to those obtained following the infusion of ascorbate (50 μ M, >120 min). The effects of 50 μ M ascorbate were also assessed on vasodilatation caused by a single dose of the nitric oxide donor, glyceryl trinitrate (10 nmol). The effect of the IK_{Ca} blocker, charybdotoxin (50 nM) was also assessed on vasodilatation induced by DCEBIO.

2.1.2.6 Effect of preventing the concentration of ascorbate on EDHF-mediated vasodilatation

As the ciliary body of the eye is known to actively transport ascorbate (Millar & Kaufman, 1995), it is possible that accumulation of ascorbate in the aqueous humour at a concentration greater than that infused (50 μ M), is a prerequisite for its ability to block vasodilator responses. This hypothesis was assessed in a series of experiments where the ability of the eye to accumulate ascorbate was impaired. Firstly, as the ciliary body secretes ascorbate into the posterior chamber of the eye (figure 2.2) the ability of the aqueous humour to accumulate ascorbate was prevented by continually flushing the posterior and anterior chambers with Krebs solution (lacking ascorbate) at a rate of 0.25 ml min⁻¹. This was done by inserting an inflow cannula (23G needle) through the cornea and placing it so that its tip sat between the iris and the lens in the posterior chamber. A second (outflow) cannula (26G needle) was inserted through the cornea and placed so as to sit in front of the lens and iris in the anterior chamber (figure 2.2). The second experimental approach involved making an incision in the cornea and removing

the lens, thus allowing the aqueous humour to drain freely. This prevented any possible accumulation of ascorbate by effectively destroying the posterior and anterior chambers. In these eyes, a second incision was then made around the optic nerve at the point where it inserts into the sclera. This formed an opening through which the vitreous humour was removed, producing a preparation where there were no intact ocular chambers, thereby preventing accumulation of ascorbate and removing the potential ascorbate "reservoirs" of vitreous and aqueous humour. In both these experiments the effects of ascorbate on acetylcholine-induced vasodilatation were assessed as described for the time-course experiments. It should also be noted that in the flushing experiments the aqueous and vitreous humours were sampled for assay of ascorbate (see section 2.4.4).

2.2 Perfused mesenteric arterial bed of the rat

2.2.1 Preparation of the rat isolated perfused mesenteric arterial bed

Male Wistar rats (200-350 g) were killed by concussion followed by exsanguination. The mesenteric arterial bed was then prepared using a modification of the method described by McGregor (1965). An incision was made along the abdomen and the stomach and liver were removed to aid location of the superior mesenteric artery. Once this artery was located it was cannulated *in situ* (using a cannula made from polythene tubing bore 2.5 mm, wall thickness 1 mm) at its junction with the aorta. The mesenteric arterial vasculature was then dissected away from the intestines. It was then suspended in a heating jacket, allowed to hang freely and perfused at 37°C with Krebs at a flow rate of 5 ml min⁻¹ (using the same apparatus as for bovine eye preparations). There was no need to increase the pressure in a stepwise manner, as there was little chance of damaging the microvasculature,

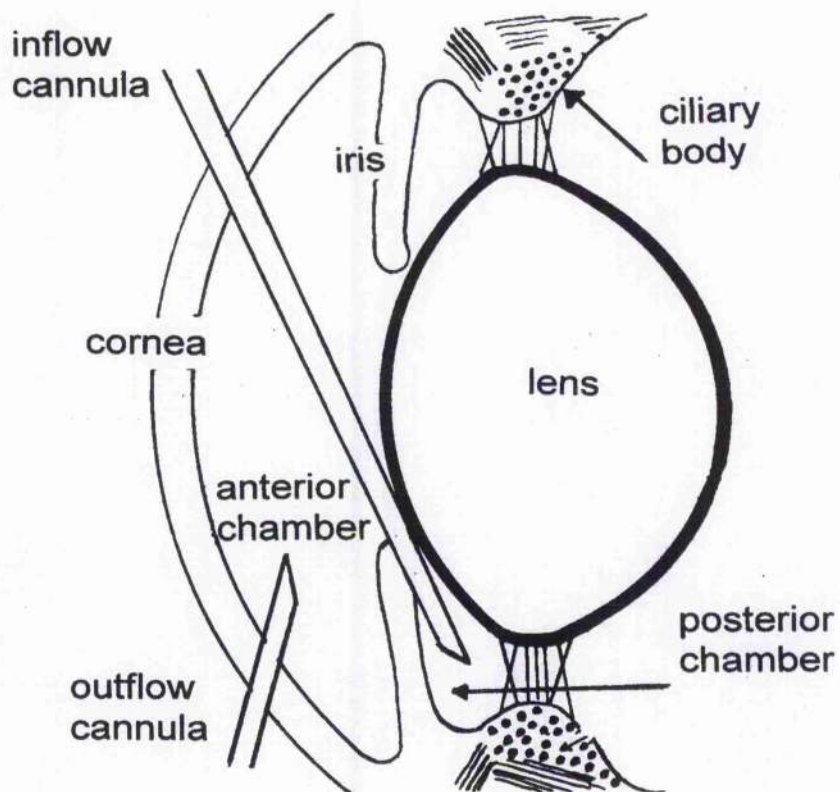


Figure 2.2 Diagram of the anterior segment of the eye showing the projection of the ciliary body into the posterior chamber and the location of cannulae during flushing experiments. The posterior chamber of the eye is defined as the aqueous-filled space behind the iris. The anterior chamber of the eye is the aqueous-filled chamber in front of the iris and lens, bound anteriorly by the cornea.

because only the arterial vessels were present. The mesenteries were then perfused for an equilibration period of at least 30 min before experiments were begun. As with the bovine cye the perfusion pressure was measured by pressure transducers *via* a side arm proximal to the infusion cannula.

2.2.2 Experimental protocols with the rat isolated perfused mesenteric arterial bed

Experiments were conducted with the rat isolated perfused mesenteric vascular bed in order to determine if the EDHF-mediated vasodilator responses to acetylcholine were also blocked by ascorbate in this preparation. In order to observe vasodilator responses, perfusion pressure was first raised to ~120 mmHg using phenylephrine (3-10 μ M). All experiments investigating the actions of ascorbate were conducted on tissues treated with L-NAME (100 μ M) and indomethacin (10 μ M) in order to block any vasodilator contribution by nitric oxide and prostanoids. Under these conditions acetylcholine-induced vasodilatation is mediated by EDHF (McCulloch *et al.*, 1997)

Control dose-response curves for EDHF-mediated vasodilatation to acetylcholine (100 fmol – 100 nmol) were constructed in mesenteries perfused with Krebs solution. These were compared with those obtained in preparations perfused with Krebs solution containing ascorbate (50 μ M, 180 min). In addition, experiments were conducted to determine the time course over which ascorbate inhibits acetylcholine-induced, EDHF-mediated vasodilatation. In order to assess their reproducibility, control vasodilator responses to acetylcholine (10 nmol) were elicited every 15 min during a 180 min period in mesenteries perfused from the outset with normal Krebs solution. When the effects of ascorbate were to be assessed, control acetylcholine-induced vasodilator responses were allowed to stabilise before addition of ascorbate (50 μ M) to the Krebs

solution. The effects of ascorbate on acetylcholine-induced vasodilatation were then assessed every 15 min during the ensuing 180 min.

2.3 Porcine coronary artery

N.B All experiments with the porcine coronary artery were conducted by my colleague Silvia Nelli in a collaborative study.

2.3.1 Preparation of porcine left anterior descending coronary artery rings

Sections of myocardium containing the left anterior descending coronary artery were cut from porcine hearts at a local abattoir and transported to the lab in Krebs solution. The left anterior descending coronary artery was dissected out, cut into 2.5 mm ring segments, suspended between two steel hooks within 10 ml organ baths and maintained at 37°C in Krebs solution gassed with O₂ containing 5% CO₂. Some tissues were not used on the day they were obtained, in which case they were stored in Krebs solution at 4°C and used the next day. The tension was recorded isometrically using Grass FT03C transducers and displayed and recorded using a computerised data acquisition system (MacLab/8e, ADInstruments). The resting tension was adjusted to 2g and tissues were allowed to equilibrate for 60 min before proceeding with experimental protocols, during which time the tension was readjusted to 2 g, if necessary.

2.3.2 Experimental protocols with the porcine left anterior descending coronary artery

In order to observe vasodilator responses, the rings of coronary artery were contracted to about 60% (6.6 ± 0.5 g) of the maximal U46619-induced tone using concentrations of 10-100 nM. Some of the blocking agents used, namely charybdotoxin and L-NAME,

enhanced the U46619-induced tone. Consequently, when these drugs were used, the concentration of U46619 was adjusted to ensure that the level of tone was similar to that of control experiments.

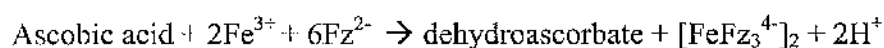
Vasodilatation to bradykinin in the porcine coronary artery has a nitric oxide-mediated and an EDHF-mediated component. Therefore, to assess EDHF-mediated vasodilatation to bradykinin, the nitric oxide synthase inhibitor, L-NAME (100 μ M), and the cyclooxygenase inhibitor, indomethacin (3 μ M) were present throughout all experiments.

In control rings, EDHF-dependent vasodilations were elicited by the addition of bradykinin (0.1– 300 nM). The effects of the small conductance calcium-sensitive potassium channel (SK_{Ca}) inhibitor, apamin (100 nM), and the large and intermediate conductance calcium-sensitive potassium channel (BK_{Ca}/IK_{Ca}) inhibitor, charybdotoxin (100 nM), each alone and in combination were assessed. Apamin and charybdotoxin were present for at least twenty min before effects on vasodilator responses were tested.

The effects of ascorbate were also assessed on EDHF-mediated, bradykinin-induced vasodilatation in the presence and absence of the SK_{Ca} and BK_{Ca}/IK_{Ca} blocking drugs apamin and charybdotoxin (both 100 nM), each alone and in combination. In these experiments ascorbate (150 μ M) was included in the Krebs solution used to bathe the arterial rings and was present for at least two hours before vasodilator responses were elicited.

2.4 Spectrophotometric assay of ascorbate

In some experiments samples (~1.5 ml) of aqueous and vitreous humour were removed from bovine eyes using a syringe and their ascorbate content was measured using a modification of an established spectrophotometric assay for ascorbate (Ortega-Barrales *et al.*, 1998; Pascual-Reguera *et al.*, 1997). The basis of this assay is that ascorbate can reduce Fe(III) to Fe(II), which reacts with ferrozine in acidic conditions (see below) to produce a magenta coloured product (Pascual-Reguera *et al.*, 1997).



2.4.1 General procedure

0.5 ml of samples or authentic ascorbate standards (2.5-500 μM) were added to centrifuge tubes. To these, 0.5 ml volumes of Fe(III)Cl₃ (1 mM), sodium ferrozine (10 mM) and hexamethylenetetramine buffer (HMTA, 0.23 M, pH 5), were added. The final volume was adjusted to 5 ml with distilled water and the samples were mixed by vortexing. The tubes were incubated at 25°C for 10 min, then centrifuged at 100 g for 15 min at 4°C (IEC Centra-8R). Absorbance was then measured at 562 nm using a spectrophotometer (Pyc Unicam, model SP6-550). Authentic ascorbate standards were prepared from a 100 mM stock that was prepared daily. The HMTA buffer was prepared by dissolving 3.5 g in 4 M HCl and made to a final volume of 100 ml with distilled water and the pH of the buffer was adjusted to 5 by addition of a few drops of NaOH (4 M). The buffer is only stable for a week at 4°C (Ortega-Barrales *et al.*, 1998), therefore a solution was made weekly. The ferrozine solution is stable for a month at 4°C (Ortega-Barrales *et al.*, 1998), therefore fresh ferrozine solution was prepared at least once a month.

2.4.2 Standard curve and absorption spectra

Experiments were first conducted to assess the absorption spectrum of the magenta Fe(II)/ferrozine complex formed in the assay, when ascorbate (50 μ M) was present, using a dual-beam scanning spectrophotometer (Shimadzu, UV-240IPC). The single absorbance peak was maximal at 562 nm (range of scan: 400-800 nm), confirming that indicated by Pascual-Reguera *et al* (1997; Figure 2.3). Experiments were performed to ascertain the upper and lower detection limits of the assay and the linearity of the standard curve (figure 2.4). In these, known concentrations of ascorbic acid (2.5-500 μ M, 0.5 ml) were added to the assay mixture. The limit of detection was \sim 5-25 μ M ascorbate and the standard curve linear to 500 μ M (figure 2.3). If absorbance of samples exceeded 1 absorbance unit, they were diluted with distilled water to bring them into the linear range; the absorbance value was then multiplied by the dilution factor.

2.4.3 Measurement of ascorbate concentration from samples of vitreous and aqueous humours

Samples of vitreous humour were obtained by inserting a needle (19G) into the sclera toward the rear of the eye and removing about 1.5 ml with a 2.5 ml syringe. Care was taken to place the needle to minimise the risk of removal of retinal or other cellular tissue. When sampling aqueous humour, each sample was obtained from both the posterior and anterior chambers, as it is reported that there is a slight difference in ascorbate concentration in each chamber (Davson, 1980). The aqueous humour was collected by piercing the cornea with a needle (23G) and placing it in the posterior chamber, where some aqueous (\sim 0.75 ml) was removed with a 2.5 ml syringe. The

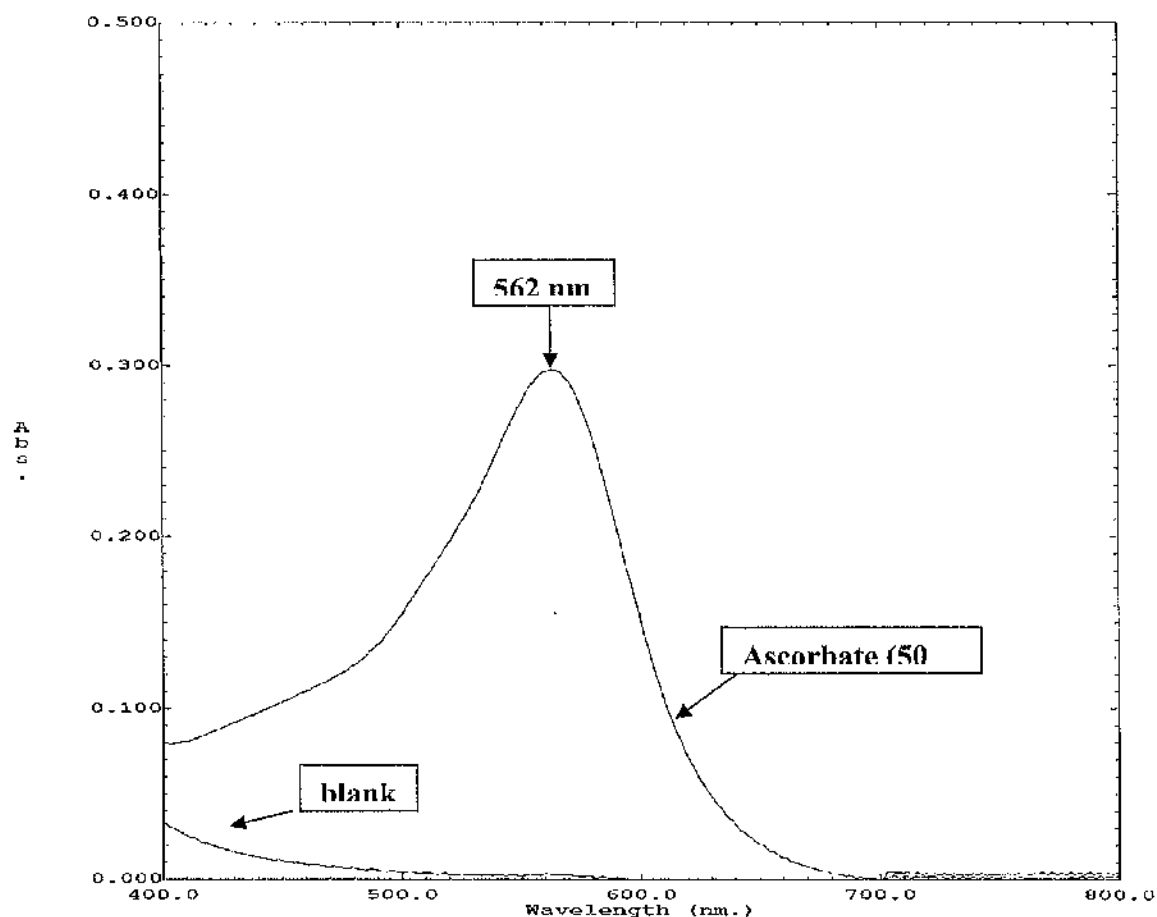


Figure 2.3 Absorbance (Abs) spectrum (400-800 nm) for the magenta Fe(II)/ferrozine complex formed in the presence of a single concentration of authentic ascorbate (50 μ M). At this concentration of ascorbate the assay produced a single large absorbance peak, which was maximal at 562 nm. A scan of a blank containing all assay chemicals but no ascorbate shows that there was no significant absorbance, at any wavelength above 500 nm.

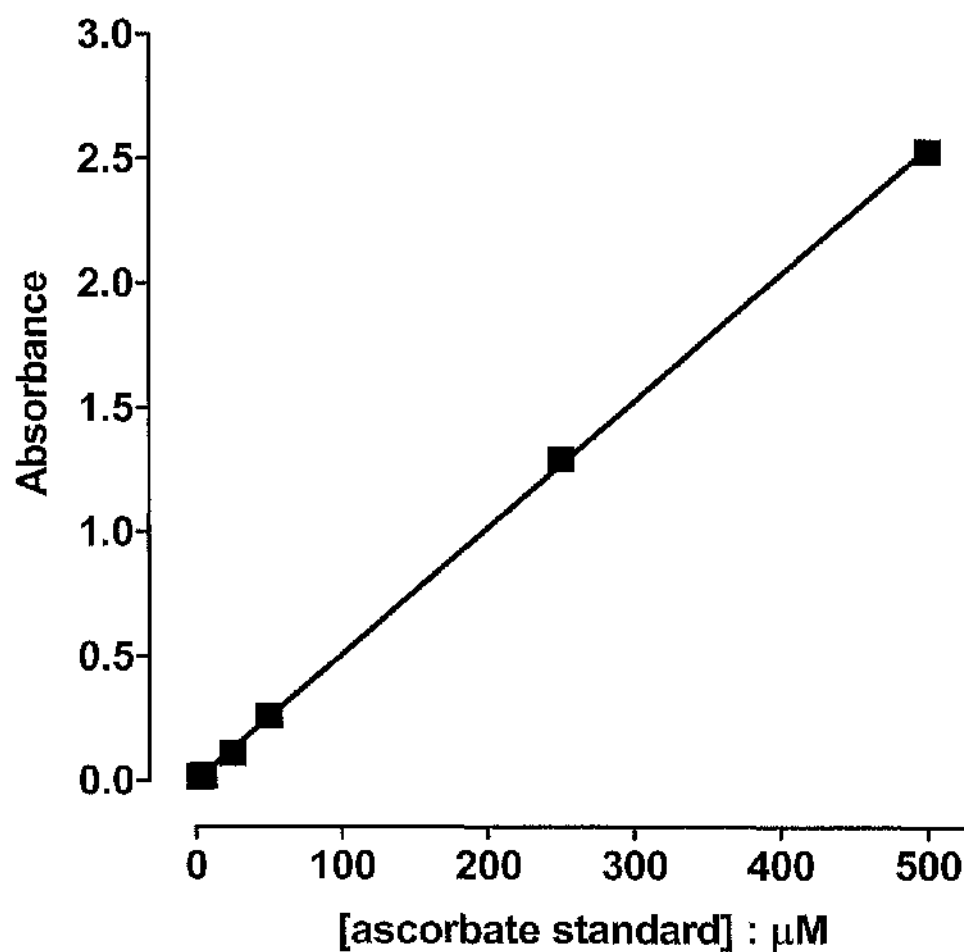


Figure 2.4 Standard curve for the spectrophotometric measurement of ascorbate. The graph shows the absorbance produced by known concentrations of ascorbate (2.5 – 500 μM) under the assay conditions described in section 2.4.2. N.B. the absorbance values over one absorbance unit were calculated from diluted standard samples.

needle was then relocated to the anterior chamber and an equal volume aqueous removed to give a final sample volume of about 1.5 ml. As the concentration of ascorbate was expected to be around 1 mM (Davson, 1980; Halliwell & Gutteridge, 1989) the samples of both aqueous humour and vitreous humour were diluted (1:10) with saline (0.9%), to give an absorbance value which would fall in the linear part of the standard curve; the absorbance value obtained was then multiplied to correct for the dilution. In order to validate that the absorbance levels measured in samples were truly reflective of the presence of ascorbate, experiments were conducted to determine if they were affected by ascorbate oxidase (1.5 u ml^{-1} , 20 min, 25°C). In these experiments the absorbance value obtained from the diluted samples was then compared to an equivalent concentration of standard ascorbate ($100 \text{ }\mu\text{M}$) that had also been incubated with ascorbate oxidase (figure 2.5). As degree of the reduction of absorbance by ascorbate oxidase was similar in both samples and authentic ascorbate standards, it was considered that the signal did indeed represent ascorbate. The inability of ascorbate oxidase to fully digest all the ascorbate present, even in standards, may result from end product inhibition; it is unlikely to be due to insufficient incubation time or the amount of ascorbate oxidase used, longer incubation times or as higher concentrations of enzyme had no further effect on the ascorbate signals generated (data not shown).

2.5 Drugs and solvents

Acetylcholine chloride, ascorbic acid, ascorbate oxidase (from *Cucurbita sp.*), apamin (from bee venom), atropine sulphate, barium chloride, bradykinin acetate, catalase (from bovine liver), CHAPS (3-[(cholamidopropyl)dimethyl-ammonio]1-propane sulphonate), dehydroascorbate, dithiothreitol, DMSO (dimethyl sulfoxide) EDTA (ethylenediamine-tetraacetic acid disodium salt), glutathione,

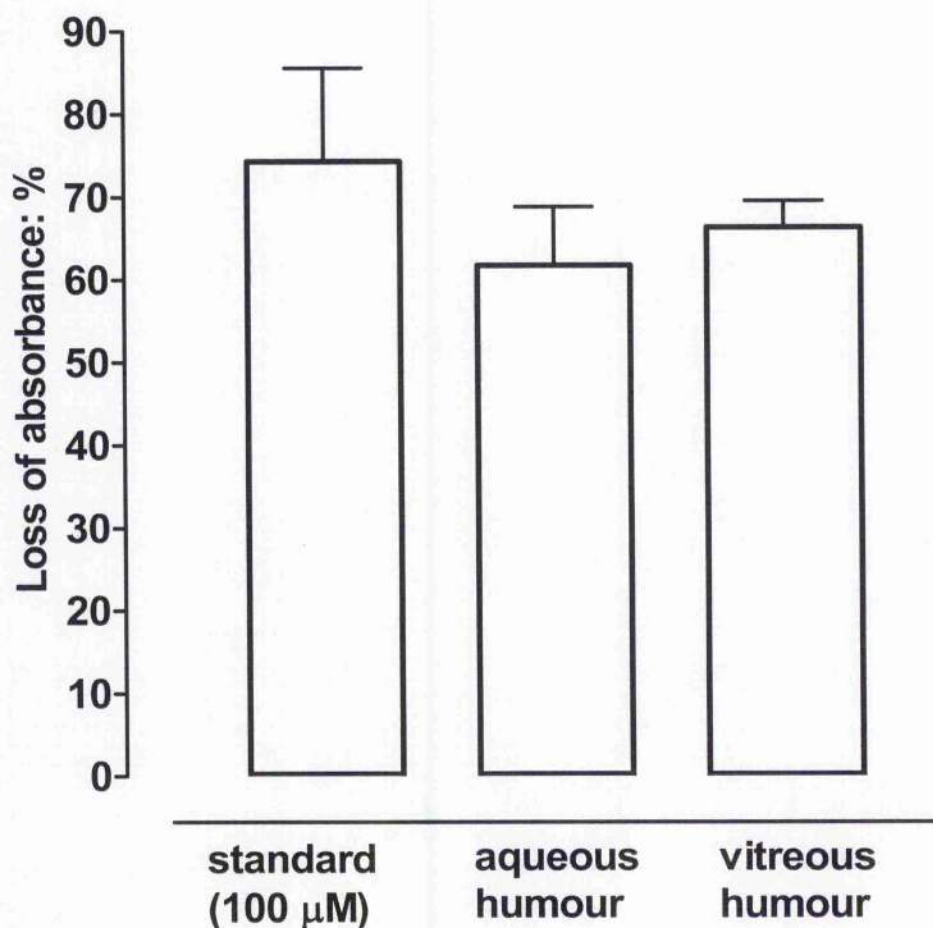


Figure 2.5 Histogram showing the digestion of ascorbate in authentic ascorbate standards and samples by ascorbate oxidase (1.5 u ml^{-1} , 20 min, 25°C). As the samples of aqueous and vitreous humour were expected to have a concentration of $\sim 1 \text{ mM}$ they were diluted 1:10 with saline (0.9 %) before being added to the reaction mixture to give an absorbance value fell within the linear part of the standard curve. For comparison, a concentration of authentic ascorbate ($100 \text{ } \mu\text{M}$) that would be similar to those in the diluted samples was incubated with ascorbate oxidase.

hexamethylenetetramine (HMTA), 5-hydroxytryptamine (5-HT) creatinine sulphate complex, indomethacin, L-NAME (N^G -nitro-L-arginine methyl ester), N-acetyl-L-cysteine, noradrenaline hydrochloride, NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one), ouabain, papaverine (free base), phenylephrine hydrochloride, prostaglandin $F_{2\alpha}$, sodium ferrozine (3-(2-(pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine), superoxide dismutase (from bovine erythrocytes), and U46619 (9,11-dideoxy-11 α ,9 α -epoxy-methanoprostaglandin $F_{2\alpha}$) were all purchased from Sigma (Poole, UK). Charybdotoxin (synthetic) and iberiotoxin (synthetic) were purchased from Latoxan (Valence, France). ODQ (1-H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) was purchased from Alexis (Nottingham, U.K.). Anandamide (water soluble emulsion), DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) and 1-EBIO (1-ethyl-2-benzimidazolinone) were all purchased from Tocris (Avonmouth, UK). Flurbiprofen was a gift from the Boots Pure Drug Company (Nottingham U.K.). Glyceryl trinitrate (10 % w w⁻¹ in lactose) was a gift from NAPP laboratories (Cambridge, UK). Glibenclamide was a gift from Hoechst (Hounslow, U.K.). Levromakalim was a gift from GlaxoSmithKline (Harlow, U.K.). All drugs were dissolved and diluted in 0.9 % saline except: DCEBIO (100 mM stock), glibenclamide (3 mM stock) and levromakalim (10 mM stock) which were dissolved in 70 % ethanol; U46619 (1 mM stock) was dissolved in 50 % ethanol; papaverine (100 mM stock) and 1-EBIO (100 mM stock) were dissolved in DMSO; 5-HT (10 mM stock) and ferric chloride hexahydrate (100 mM stock) were dissolved in HCl (10 mM); and indomethacin (10 mM stock) was dissolved in Na₂CO₃ (1 M) solution.

2.6 Statistical analysis

All results are expressed as mean \pm s.e.mean of n separate observations; n or the number of observations represent the number of eyes studied, except for data from the rat mesentery and porcine coronary artery where n corresponds to number of animals and the number of arterial rings, respectively. Vasoconstrictor responses are given in mmHg or as percentage (%) increase of U46619-induced perfusion pressure. Vasodilator responses are expressed as percent (%) reduction of U46619- (bovine eye) or phenylephrine-induced (rat mesentery) perfusion pressure or U46619-induced tone (porcine coronary artery). Graphs were drawn and statistical comparisons were made using Student's t-test, either paired or unpaired, or one-way analysis of variance with Bonferroni's post-test, as appropriate, with the aid of a computer program, Prism (GraphPad, San Diego, USA). A probability (P) less than or equal to 0.05 was considered significant.

Chapter 3

Results: 1

Characterisation of endothelium-derived hyperpolarising factor (EDHF)-like vasodilations in the ciliary vascular bed of the bovine eye



In order to characterise endothelium-dependent vasodilatation in the bovine eye it was first necessary to investigate how the ciliary vascular bed behaves under basal perfusion conditions, factors such as intrinsic tone or basal release of vasodilator substances could affect the conditions used in later experiments. It was also necessary to try and optimise the experimental conditions, such as the vasoconstrictor and vasodilator agonists used, in order to obtain the best data in subsequent experiments.

3.1 Characterisation of basal perfusion pressure

The basal perfusion pressure of the ciliary vascular bed of the bovine eye at a constant flow of 2.5 ml min^{-1} was $31.8 \pm 1.5 \text{ mmHg}$ ($n = 105$). Infusion of a general smooth muscle relaxant, the non-selective inhibitor of phosphodiesterases, papaverine ($150 \text{ }\mu\text{M}$, 20 min), caused a small but significant fall in this basal perfusion pressure (change of $-5.0 \pm 1.0 \text{ mmHg}$, $n = 5$, $P < 0.01$, paired t-test; Figure 3.1). Addition of the inhibitor of nitric oxide synthase, L-NAME ($100 \text{ }\mu\text{M}$, 20 min), to the perfusate caused no significant change in the basal perfusion pressure (change of $-0.6 \pm 3.3 \text{ mmHg}$, $n = 8$; Figure 3.1). However, the inhibitor of soluble guanylate cyclase, ODQ ($10 \text{ }\mu\text{M}$, 20 min), produced a small but significant rise in the basal perfusion pressure ($14.6 \pm 3.6 \text{ mmHg}$, $n = 10$, $P < 0.01$, paired t-test; Figure 3.1).

3.2 Characterisation of responses to vasoconstrictor agonists

Concentration-response curves were constructed in the ciliary vascular bed of the bovine eye for a number of agonists commonly used to induce vasoconstrictor responses. These drugs were; the thromboxane A_2 mimetic, U46619; prostaglandin $F_{2\alpha}$; the endogenous adrenoceptor agonist, noradrenaline; the synthetic α_1 -adrenoceptor

agonist, phenylephrine and 5-HT. Each of these drugs produced concentration-dependent rises in perfusion pressure (Figure 3.2a and table 3.1).

As will be seen in the Discussion (Chapter 5) only noradrenaline, 5-HT and U46619 were used for further study. A single concentration of each vasoconstrictor was infused constantly to cause a sub-maximal rise in perfusion pressure; the concentration of each vasoconstrictor was chosen to give a similar perfusion pressure in each experiment (Table 3.2). Additional infusion of the nitric oxide synthase inhibitor, L-NAME (100 μ M) caused a significant increase in the sub-maximal perfusion pressure for all three vasoconstrictor agonists (Table 3.2). In experiments to assess vasodilator responses, the perfusion pressure was raised with concentrations of noradrenaline, 5-HT, or U46619 which produced a submaximal vasoconstriction. In these experiments, bolus injections of acetylcholine (1 pmol – 10 nmol) produced dose-dependent falls in perfusion pressure (Figure 3.2b). However, the acetylcholine (10 nmol)-induced vasodilatation was greater when vasoconstriction was induced by U46619 (maximum fall of 59.5 ± 4.9 %) than when vasoconstriction was induced by noradrenaline (maximum fall of 29.0 ± 5.2 %) or 5-HT (maximum fall of 10.5 ± 4.6 %).

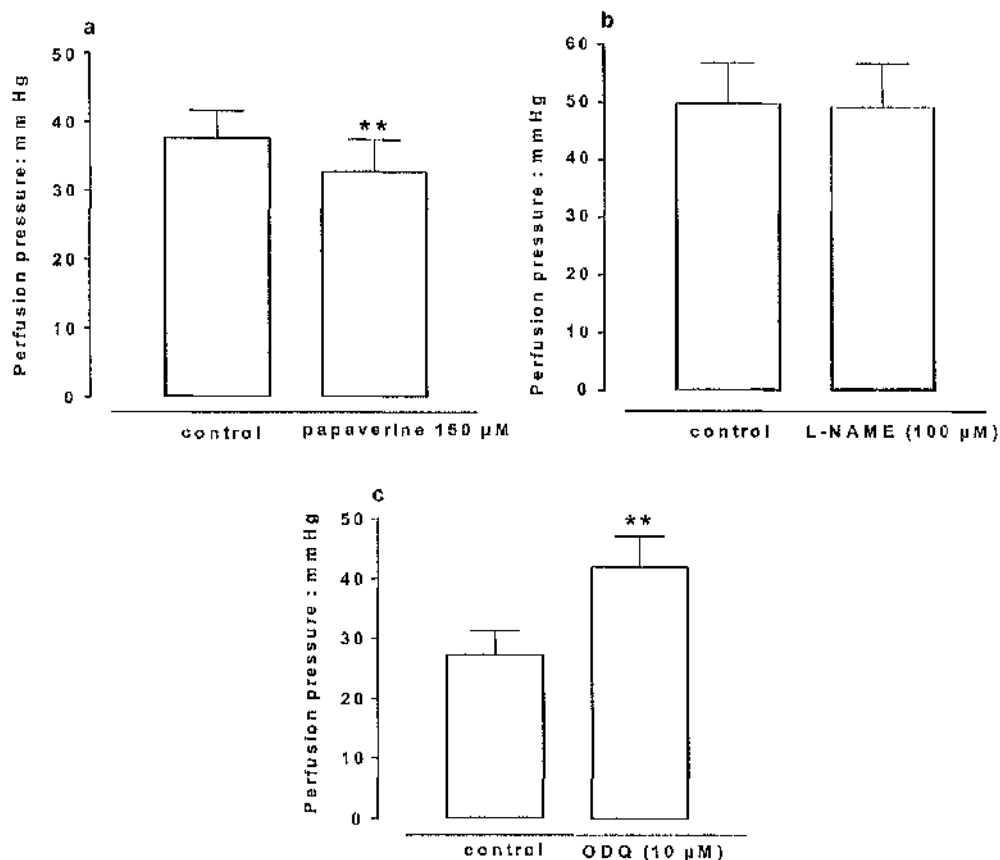


Figure 3.1 Histograms showing the effects a range of drugs on the basal perfusion pressure of the ciliary vascular bed of the bovine eye. (a) Effect of the non-selective phosphodiesterase inhibitor, papaverine (150 μ M). (b) Effect of the nitric oxide synthase inhibitor, L-NAME (100 μ M). (c) Effect of inhibition of soluble guanylate cyclase using, ODQ (10 μ M). Data represent the mean \pm s.e.mean of five to ten observations. ** $P < 0.01$ indicates a difference from control (paired t-test).

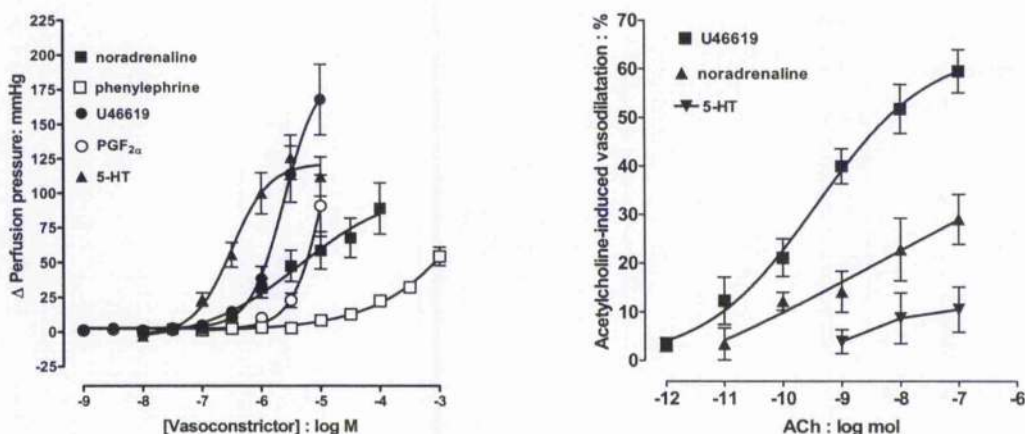


Figure 3.2 (a) Cumulative concentration-response curves showing vasoconstriction induced on the ciliary vascular bed of the bovine eye by: the endogenous adrenoceptor agonist, noradrenaline (10 nM - 100 μ M); the synthetic α_1 -adrenoceptor agonist, phenylephrine (100 nM - 1 mM); the thromboxane A₂-mimetic, U46619 (1 nM - 10 μ M); prostaglandin F_{2 α} (1 nM - 10 μ M); and 5-HT (10 nM - 10 μ M). pEC₅₀ and maximum constrictions are provided in Table 3.1. (b) Dose-response curves showing vasodilatation to acetylcholine (ACh, 1 pmol-100 nmol) in the presence of submaximal concentrations of the vasoconstrictors U46619 (maximum vasodilatation = $65.6 \pm 5.7\%$, pED₅₀ = 9.4 ± 0.2 , $n = 8$) noradrenaline (vasodilatation of 30.0 ± 5.2 at 100 nmol ACh, $n = 8$) and 5-HT (vasodilatation of $10.5 \pm 4.6\%$ with 100 nmol ACh, $n = 5$). pED₅₀ values could not be calculated for the ACh-induced vasodilations in the presence of noradrenaline and 5-HT as no maximal vasodilatation was obtained. Data represent the mean \pm s.e.mean of five to fourteen observations.

Drug	Maximum constriction: mmHg	pEC ₅₀	n
noradrenaline	102.2 ± 20.9	5.4 ± 0.3	6
Phenylephrine	54.4 ± 6.7 ⁺	N/A	14
U46619	187.6 ± 5.6	5.6 ± 0.3	6
prostaglandin F _{2α}	90.8 ± 22.3 ⁺⁺	N/A	6
5-HT	121.4 ± 7.7	6.5 ± 0.1	5

Table 3.1 The maximal vasoconstrictor response and the pEC₅₀, as predicted by GraphPad Prism, in the ciliary vascular bed of the bovine eye, for a range of vasoconstrictor agonists; the adrenoceptor agonists, noradrenaline and phenylephrine; the thromboxane A₂-mimetic, U46619; prostaglandin F_{2α}; and 5-HT. N.B Maximal contractions could not be obtained for phenylephrine or prostaglandin F_{2α} the stock concentrations required were insoluble, therefore Prism could not calculate pEC₅₀ values were not calculated for these drugs. ⁺The constriction induced by phenylephrine at a concentration of 1mM. ⁺⁺The constriction induced by prostaglandin F_{2α} at a concentration of 10 μM. Data are expressed as the mean ± s.e.mean of five to fourteen observations.

Drug	Perfusion pressure; mmHg	Perfusion pressure + L-NAME: mmHg	n
noradrenaline	98 ± 15.4	134.3 ± 19.7*	4
5-HT	107.7 ± 9.3	151.6 ± 10.4*	6
U46619	139.2 ± 26.8	163.0 ± 27.5*	8

Table 3.2 Table showing the effect of infusion of the nitric oxide synthase inhibitor, L-NAME (100 µM) on perfusion pressure induced by noradrenaline (100 µM), 5-HT (300 nM) and U46619 (100 nM) in the ciliary vascular bed of the bovine eye. Data represent the mean ± s.e.mean of four to eight observations. * $P < 0.05$ represents a significant increase in perfusion pressure in the presence of L-NAME (paired t-test).

3.3 Endothelial dependence of vasodilator responses to acetylcholine and bradykinin and effects of L-NAME, ODQ and flurbiprofen

As indicated in Chapter 3, all further experiments in the bovine eye were conducted using U46619 as the vasoconstrictor agent, as bigger vasodilator responses to acetylcholine could be elicited when using this drug. Following infusion of U46619 (50-500 nM), which increased the perfusion pressure to 131.8 ± 16.3 mmHg ($n = 8$), bolus injections of acetylcholine (1 pmol-100 nmol) immediately proximal to the preparation produced dose-dependent falls in the perfusion pressure ($pED_{50} = 9.42 \pm 0.16$, maximum fall of 64.1 ± 4.3 %, Figure 3.3a, 3.4a). As L-NAME (100 μ M, 20 min) and ODQ (10 μ M, 20 min) enhanced the U46619-induced perfusion pressure (see sections 3.1, 3.2), when experiments were conducted with these agents the concentration of U46619 was adjusted such that the final perfusion pressures obtained were not significantly different to control preparations (134.9 ± 10.9 mmHg, $n = 9$, and 116.4 ± 17.3 mmHg, $n = 5$, respectively). Under these conditions, neither L-NAME (Figure 3.3b, 3.4a) nor ODQ (Figure 3.4a) had any effect on the vasodilator actions of acetylcholine. Furthermore, the fall in perfusion pressure induced by 10 nmol acetylcholine (45.6 ± 3.2 %, $n=18$) was unaffected by the cyclo-oxygenase inhibitor, flurbiprofen (30 μ M, 20 min) alone, nor by the combination of flurbiprofen and L-NAME (Figure 3.5a).

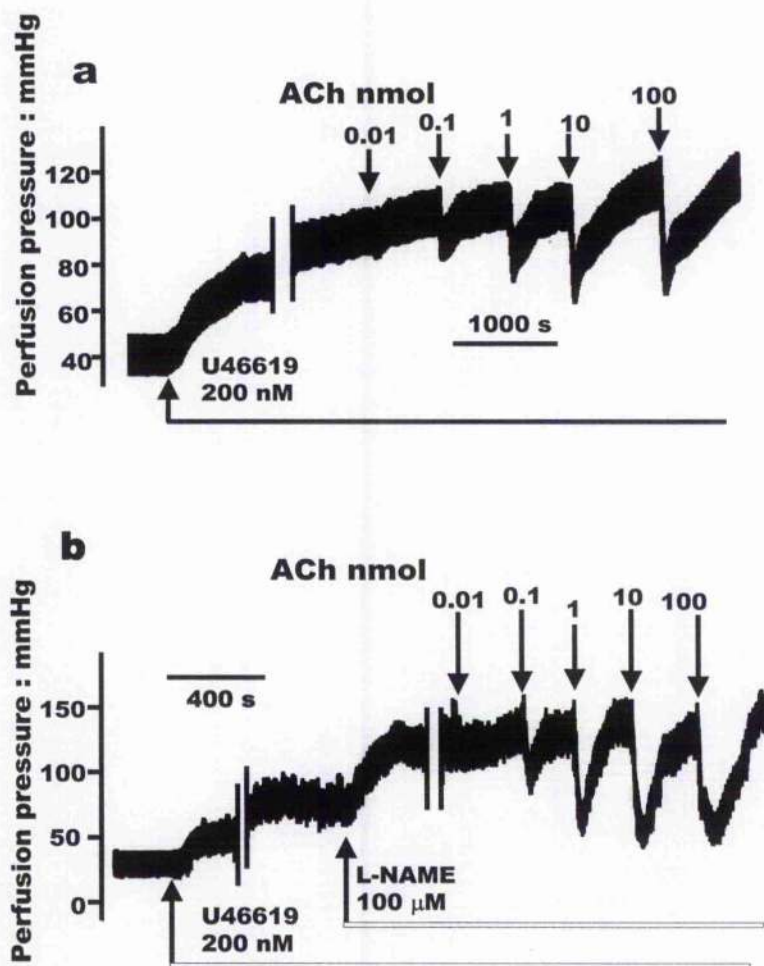


Figure 3.3 Original traces showing acetylcholine-induced vasodilatation in the bovine isolated perfused eye. The perfusion pressure was increased using the thromboxane A₂-mimetic, U46619 (200 nM), prior to addition of graded doses of ACh. (a) Dose-dependent vasodilatation produced by ACh (0.01-100 nmol). (b) Addition of the nitric oxide synthase inhibitor, L-NAME (100 μM), produced vasoconstriction but had no effect on ACh-induced vasodilatation.

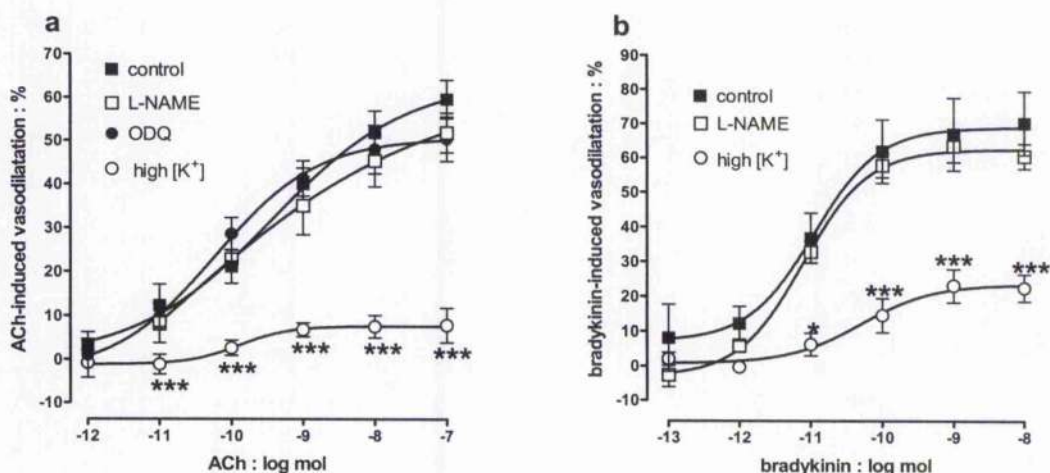


Figure 3.4 Control dose-response curves showing vasodilatation induced by bolus doses of (a) ACh (control $R_{\max} = 65.6 \pm 5.7$ %, $pED_{50} = 9.4 \pm 0.2$; 1 pmol–100 nmol) and (b) BK (control $R_{\max} = 69.1 \pm 5.7$ %, $pED_{50} = 10.9 \pm 0.05$; 100 fmol–10 nmol) on the bovine isolated perfused eye preparation. The effects of treatment with the nitric oxide synthase inhibitor, L-NAME (100 μ M, ACh: $R_{\max} = 63.1 \pm 4.6$ %, $pED_{50} = 10.9 \pm 0.9$; BK: $R_{\max} = 62.4 \pm 1.5$ %, $pED_{50} = 11.1 \pm 0.1$), the inhibitor of soluble guanylate cyclase, ODQ (10 μ M, ACh: $R_{\max} = 50.6 \pm 1.2$ %, $pED_{50} = 10.3 \pm 0.1$), or a high concentration of KCl (30 mM, ACh: $R_{\max} = 7.6 \pm 0.3$ %, $pED_{50} = 9.9 \pm 0.1$; BK: $R_{\max} = 23.4 \pm 2.0$ %, $pED_{50} = 10.3 \pm 0.2$) are also shown. Data represent the mean \pm s.e.mean of five to twelve observations. * $P < 0.05$ and *** $P < 0.001$ indicate a difference from control.

Bradykinin (0.1 pmol–10 nmol) also produced dose-dependent falls in U46619-induced perfusion pressure ($pED_{50}=10.95 \pm 0.05$, maximum fall of $68.8 \pm 1.1\%$, $n = 6$, Figure 3.4b). In the presence of L-NAME (100 μ M, 20 min), bradykinin-induced falls in perfusion pressure were completely unaffected (Figure 3.4b). In addition, the fall in perfusion pressure produced by 10 pmol bradykinin ($47.0 \pm 4.4\%$, $n = 15$) was unaffected by flurbiprofen alone, nor by the combination of flurbiprofen and L-NAME (Figure 3.5b).

Infusion of the detergent CHAPS (0.3 %, 2 min), in order to selectively damage the endothelium (Randall & Hiley, 1988), had no effect on U46619-induced perfusion pressure (160.0 ± 16.4 and 170.2 ± 16.6 mmHg, $n = 13$, before and after CHAPS, respectively), but resulted in a profound reduction in the fall in perfusion pressure induced by 10 nmol acetylcholine or 10 pmol bradykinin (Figure 3.5a, 3.5b).

3.4 Effect of high $[K^+]$ on vasodilator responses to acetylcholine and bradykinin

The ability of acetylcholine and bradykinin to induce vasodilator responses in the bovine eye was examined in the presence of Krebs solution containing a high concentration of KCl (30 mM). In these experiments KCl itself produced a sustained increase in perfusion pressure to 98.7 ± 7.1 mmHg ($n = 12$), but in order to examine vasodilator responses at levels of pressure similar to control experiments the pressure was increased to 137.6 ± 12.3 mmHg ($n = 12$) with the infusion of U46619 (5–10 nM). Under these conditions, vasodilator responses to acetylcholine (1 pmol–100 nmol) and to bradykinin (0.1 pmol–10 nmol) were inhibited (Figure 3.4a, 3.4b).

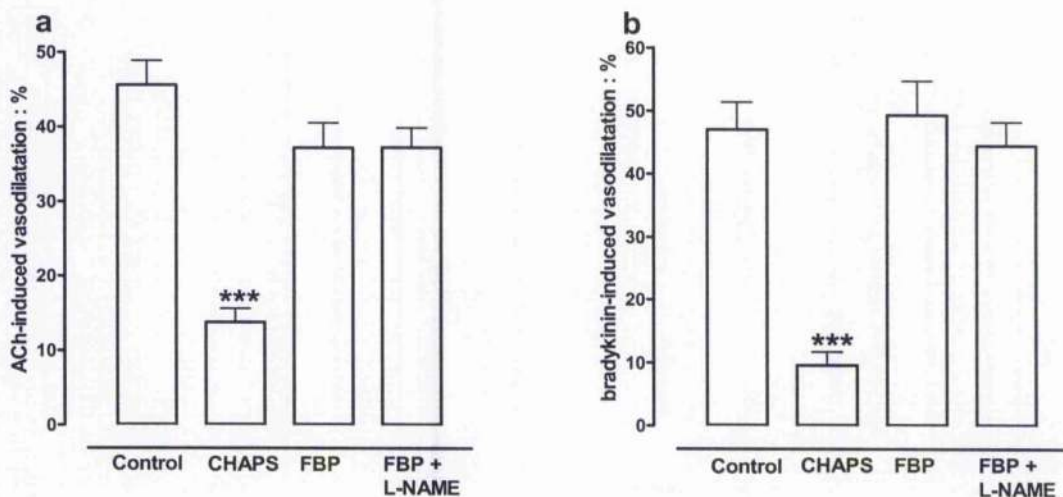


Figure 3.5 Histogram showing the effects of damaging the endothelium in the ciliary vascular bed of the bovine eye with CHAPS (0.3%, 2 min), inhibiting cyclooxygenase with flurbiprofen (FBP, 30 μ M) and combined treatment with flurbiprofen and the inhibitor of nitric oxide synthase, L-NAME (100 μ M), on vasodilatation induced by (a) ACh (10 nmol) or (b) BK (10 pmol). Data represent the mean \pm s.e.mean of ten to eighteen observations. *** $P < 0.001$ indicates a difference from control.

3.5 Effect of K⁺ channel blockers and ouabain on acetylcholine-induced vasodilatation

A number of agents that block specific potassium channels were infused in the bovine eye for 20 min and their effects on acetylcholine (10 nmol)-induced vasodilator responses assessed. The K_{ATP} channel blocker, glibenclamide (10 μ M), had no effect on U46619-induced perfusion pressure (104.7 ± 7.1 mmHg before and 106.1 ± 9.6 mmHg after infusion; $n = 10$) or on acetylcholine-induced vasodilatation (Figure 3.6a). In contrast, the non-selective K_{Ca} channel blocker, TEA (10 mM), produced a large transient increase in U46619-induced perfusion pressure (291.9 ± 57.5 mmHg, $n = 4$) which returned to a perfusion pressure similar to that seen before the addition of TEA after ~15 min. TEA also produced a significant inhibition of acetylcholine-induced vasodilatation (Figure 3.6a).

The K_{IR} channel blocker, barium (30 μ M), had no effect on the U46619-induced perfusion pressure (128.2 ± 8.0 mmHg, before and 118.4 ± 7.8 mmHg, after infusion; $n = 13$ and 11, respectively) or on acetylcholine-induced vasodilatation (Figure 3.6b). The Na/K ATPase inhibitor, ouabain (10 μ M) caused a slow, small but transient rise in perfusion pressure, which returned to normal (131 ± 11.6 mmHg, $n = 18$) after about 1 hour. Ouabain also produced significant blockade of acetylcholine-induced vasodilatation (Figure 3.6b). The combination of barium and ouabain produced a greater blockade of acetylcholine-induced vasodilatation than was seen with ouabain alone (Figure 3.6b). This combination had no further effect on the U46619-induced perfusion pressure than ouabain alone.

The SK_{Ca} channel blocker, apamin (100 nM), had no significant effect on U46619-induced perfusion pressure (154.8 ± 18.6 mmHg before, and 172.8 ± 11.2 mmHg after, $n = 9$) but the IK_{Ca}/BK_{Ca} inhibitor, charybdotoxin (50 nM; Figure 3.7), and the BK_{Ca} channel inhibitor, iberiotoxin (50 nM), each produced a large transient vasoconstriction (rise in perfusion pressure of 339.6 ± 18.8 mmHg, $n = 8$, and 298.5 ± 38.1 mmHg, $n = 7$, respectively) that rapidly returned (~ 15 min) to a similar perfusion pressure to that seen before exposure to the drugs (Figure 3.7). Neither apamin (100 nM) nor iberiotoxin (50 nM) alone had any significant effect on acetylcholine (10 nmol)-induced vasodilatation (Figure 3.8b), whereas charybdotoxin (50 nM) produced significant blockade, and uncovered a vasoconstriction (Figure 3.7, 3.8a). A lower concentration of charybdotoxin (10 nM) had no significant effect on perfusion pressure or acetylcholine-induced vasodilatation by itself, but significantly attenuated the vasodilatation when combined with apamin (100 nM, Figure 3.8a, 3.8b). In contrast, the combination of iberiotoxin (50 nM) and apamin (100 nM) had no effect on acetylcholine-induced vasodilatation (Figure 3.8b).

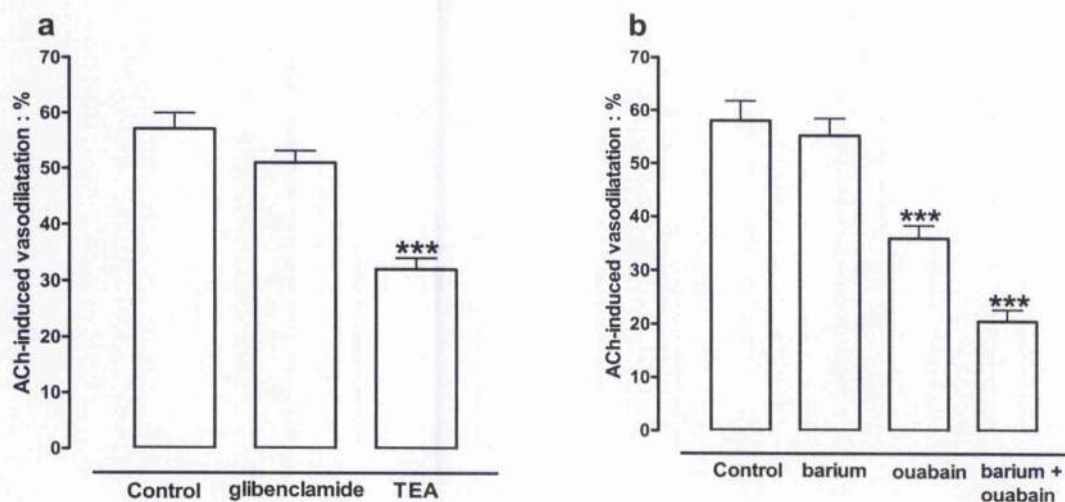


Figure 3.6 Histograms showing the effects of potassium channel blockers and of ouabain on vasodilatation induced by ACh (10 nmol) in the ciliary vascular bed of the bovine eye. (a) Effects of the K_{ATP} channel blocker, glibenclamide (10 μ M), and of the non-selective K_{Ca} channel blocker, tetraethylammonium (TEA, 10 mM). (b) Effects of the inward rectifier K channel (K_{IR}) blocker, barium (30 μ M), and of the Na/K ATPase inhibitor, ouabain (10 μ M), each alone and in combination. Data represent the mean \pm s.e.mean of six to eighteen observations. *** $P < 0.001$ indicates a difference from control (ANOVA with Bonferroni's post-test).

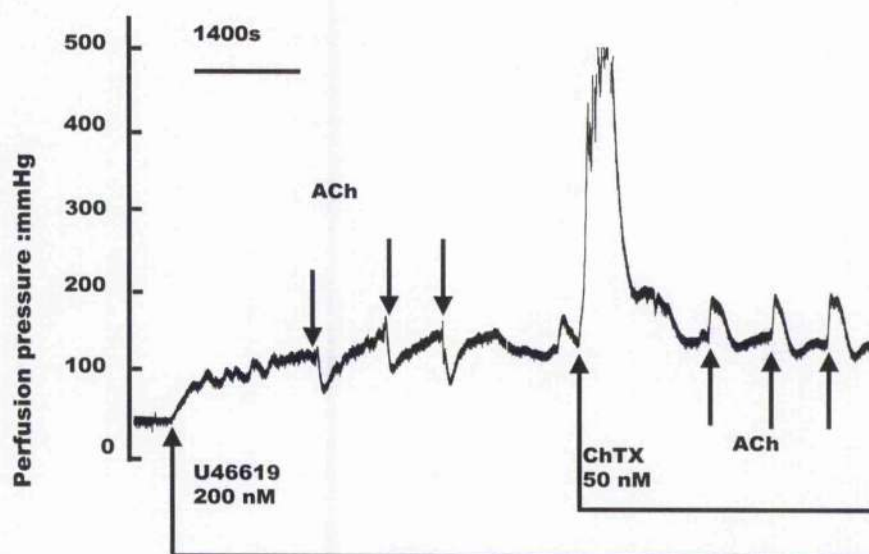


Figure 3.7 Original trace showing the effects of charybdotoxin (ChTX, 50 nM) on perfusion pressure and on vasodilatation to ACh (10 nmol) in the ciliary vascular bed of the bovine eye. The addition of charybdotoxin resulted in a large transient increase in perfusion pressure followed by reversal of ACh-induced vasodilatation to vasoconstriction.

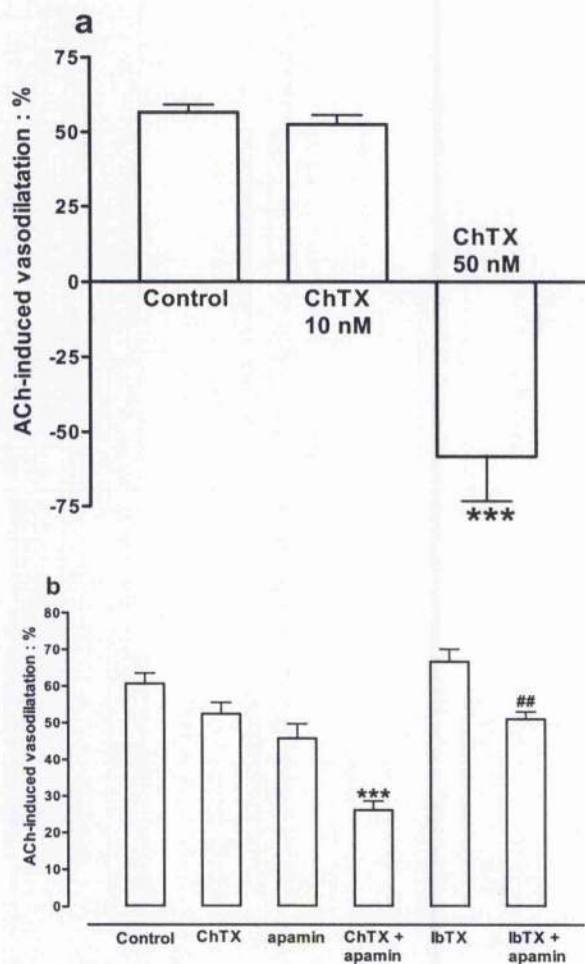


Figure 3.8 Histograms showing the effect of calcium-sensitive potassium channel inhibitors on vasodilatation induced by ACh (10 nmol) in the ciliary vascular bed of the bovine eye. (a) Effects of the large/intermediate conductance (BK_{Ca}/IK_{Ca}) calcium-sensitive K^+ channel blocker, charybdotoxin (ChTX, 10 nM and 50 nM). (b) Effects of the small conductance calcium-sensitive K^+ (SK_{Ca}) channel blocker, apamin (100 nM), alone and in combination with a low concentration of ChTX (10 nM), and of the large conductance calcium sensitive K^+ (BK_{Ca}) channel blocker, iberiotoxin (IbTX, 50 nM), alone and in combination with apamin (100 nM). Data represent the mean \pm s.e.mean of six to sixteen observations. *** $P < 0.001$ indicates a difference from control and ## $P < 0.005$ shows that the combination of iberiotoxin and apamin was different from iberiotoxin alone.

3.6 Effects of K⁺ channel openers and anandamide

The effects of a range of K⁺ channel openers commonly used to characterise or mimic EDHF were studied in the bovine eye. The opener of K_{ATP} channels, levcromakalim (0.1 – 30 nmol), produced dose-dependent vasodilations (maximum fall in perfusion pressure of 66.0 ± 7.8 %; Figure 3.9). In contrast, the putative opener of IK_{Ca}, 1-EBIO (1 nmol – 1 μ mol), produced small dose-dependent vasodilations (maximum fall in perfusion pressure of 30.0 ± 4.8 mmHg, $n = 7$; Figure 3.9). A more potent analogue of 1-EBIO, DCEBIO (100 pmol – 1 μ mol) produced dose-dependent vasodilator responses (maximum fall in perfusion pressure of 82.2 ± 6.0 mmHg, $n = 11$; Figure 3.9); however, the vasodilations produced by DCEBIO were only slightly inhibited by the infusion of charybdotoxin (50 nM, 20 min; Figure 3.9), indicating that the opening IK_{Ca} channels was not the primary cause of the vasodilator responses to this drug. Furthermore, the opener of BK_{Ca} channels, NS1619 only produced a small vasodilator response at the highest dose studied (fall in perfusion pressure of 19.1 ± 2.4 %, $n = 7$, 100 nmol). The putative candidate for EDHF, anandamide also failed to produce dose-dependent vasodilations with only the highest dose studied (100 nmol) producing a vasodilator response (fall in perfusion pressure of 21.0 ± 1.5 %, $n = 3$).

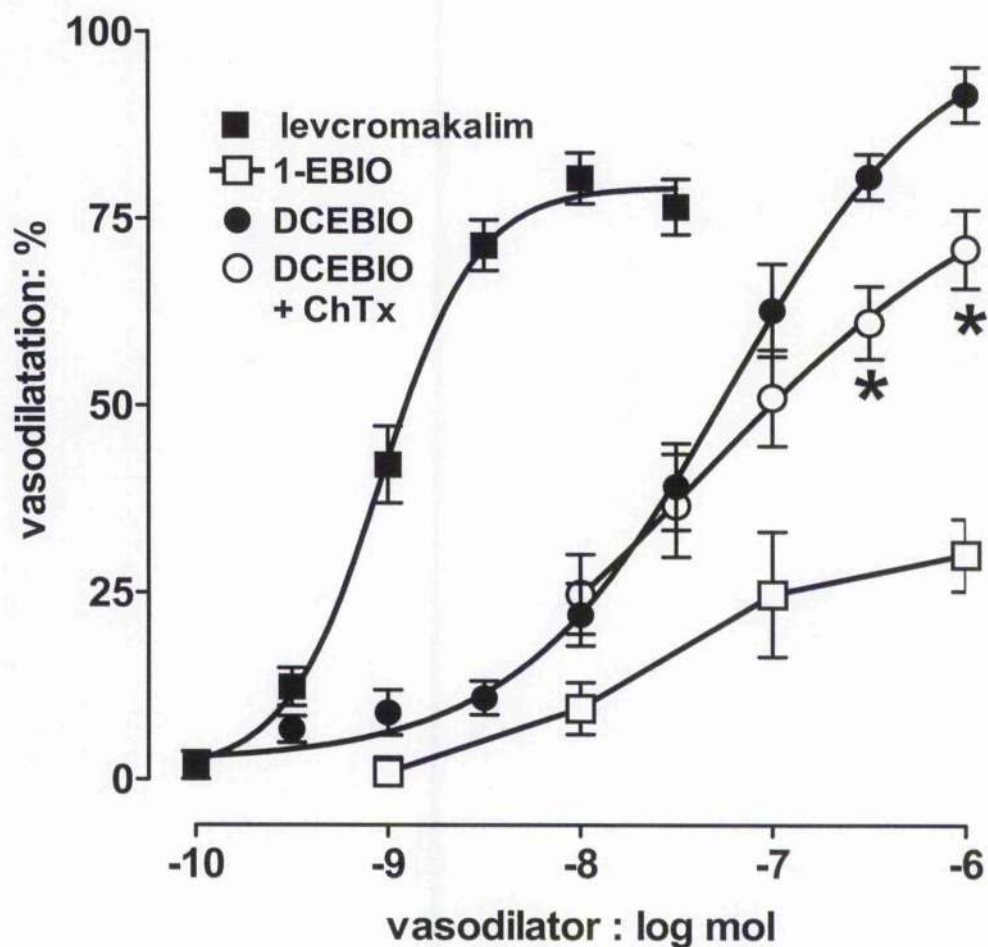


Figure 3.9 Dose-response curves showing vasodilatation in the ciliary vascular bed of the bovine eye, produced by bolus doses of a range of potassium channel openers: the opener of K_{ATP} channels, levcromakalim ($R_{max}=79.1 \pm 2.1$ %, $pED_{50}=9.0 \pm 0.04$; $0.1 - 30$ nmol); and the putative openers of IK_{Ca} , 1-EBIO (relaxation at $1 \mu\text{mol} = 30.0 \pm 4.8$ %; $1 \text{ nmol} - 1 \mu\text{mol}$) and DCEBIO ($R_{max}=102 \pm 6.5$ %, $pED_{50}=7.2 \pm 0.1$; $100 \text{ pmol} - 1 \mu\text{mol}$). The effect of the IK_{Ca} channel inhibitor, charybdotoxin (ChTx, 50 nM), on DCEBIO-induced vasodilatation is also shown ($R_{max}=87.6 \pm 18.1$ %, $pED_{50}=7.4 \pm 0.5$). Data represent the mean \pm s.e.mean of six – ten observations. * $P<0.05$ indicates that vasodilatation-induced by DCEBIO is significantly different in the presence of ChTx.

Chapter 4

Results: 2

The effect of ascorbate on EDHF-mediated
vasodilatation



4.1 Effect of ascorbate on acetylcholine-induced, EDHF-dependent vasodilatation in the ciliary vascular bed of the bovine eye

In Chapter 3 it was demonstrated that the vasodilator responses to acetylcholine and bradykinin are mediated solely by an EDHF-like substance in the ciliary vascular bed of the bovine eye. In experiments with noradrenaline and 5-HT, ascorbate was included from the outset and acetylcholine-induced vasodilator responses appeared to be inhibited compared to experiments with U46619 where ascorbate was never included; therefore, experiments were conducted to assess the effect of ascorbate on acetylcholine-induced EDHF-mediated vasodilator responses when U46619 was used as the vasoconstrictor agonist. In these experiments when bovine isolated eyes were perfused with normal Krebs solution, acetylcholine (10 nmol) produced a large fall ($59.5 \pm 4.5 \%$, $n = 8$, figure 4.1a) in the U46619-induced perfusion pressure. In contrast, in eyes perfused with Krebs solution containing ascorbate (50 μ M, > 120 min) from the outset, acetylcholine produced a vasoconstrictor response (87.0 ± 16.8 mmHg, $n = 6$), immediately followed by a small residual vasodilator response ($17.3 \pm 6.3 \%$, $n = 6$, Figure 4.1b). This reversal of vasodilatation to vasoconstriction was similar to that previously seen with the blocker of EDHF, charybdotoxin (see chapter 3; Figure 3.7). The acetylcholine-induced vasoconstriction obtained in the presence of ascorbate was unaffected by the cyclooxygenase inhibitor, flurbiprofen (30 μ M, 20 min, Figure 4.2) but was reduced to -7.6 ± 5.3 mmHg ($n = 14$, $P < 0.001$) by the non-selective muscarinic antagonist, atropine (100 nM, 20 min, Figure 4.2); indicating that the vasoconstriction was mediated by muscarinic receptors, not release of a vasoconstrictor prostanoid. The perfusion pressure generated by U46619 (300 nM) was not, however, different in control eyes (123.3 ± 10.0 mmHg, $n = 7$) and in eyes treated with ascorbate (122.7 ± 12.0 mmHg, $n = 8$, Figure 4.1).

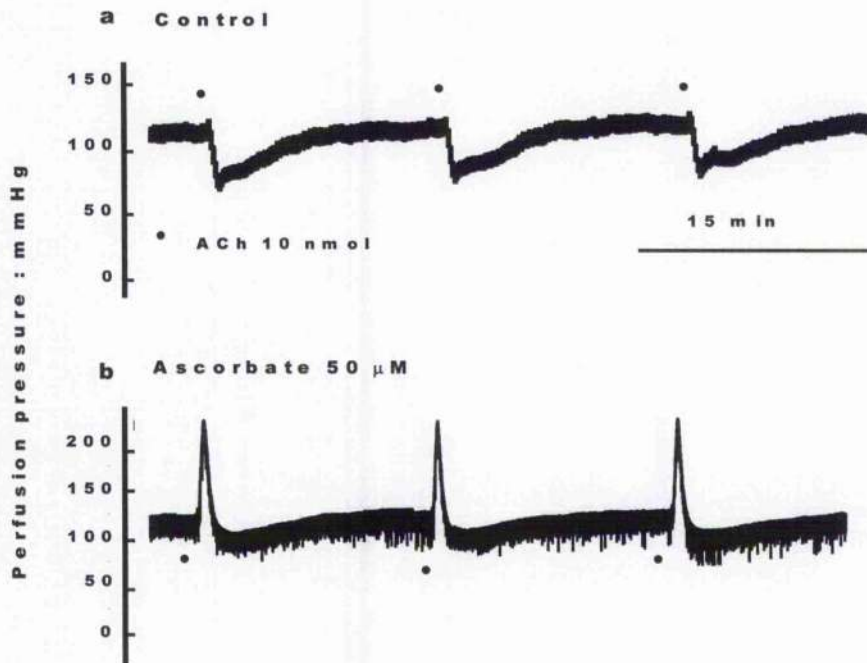


Figure 4.1 Original traces showing the difference in the responses produced by acetylcholine (ACh; 10 nmol) in the bovine isolated eye following perfusion with (a) Control Krebs solution in which acetylcholine elicited only vasodilator responses, or (b) Krebs containing ascorbate (50 μ M, 120 min) in which acetylcholine produced large vasoconstriction, followed by small residual vasodilatation.

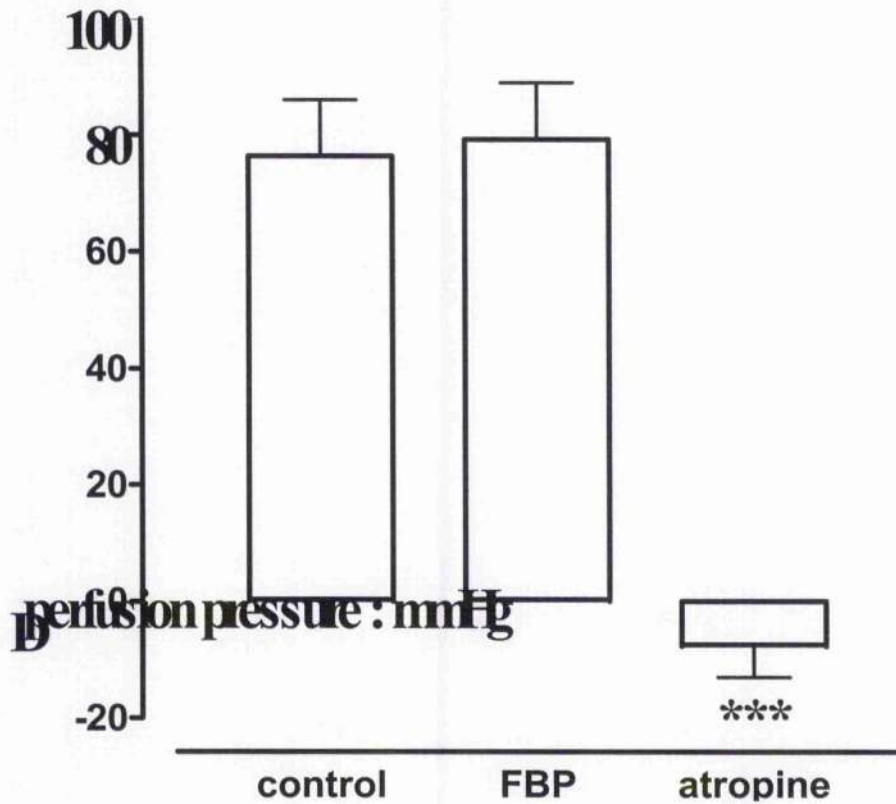


Figure 4.2 Histogram showing the effects of the cyclooxygenase inhibitor, flurbiprofen (FBP, 30 μ M), and the non-selective muscarinic receptor antagonist, atropine (100 nM), on the acetylcholine-induced vasoconstrictor response obtained in eyes perfused with Krebs containing ascorbate (50 μ M, >120 min). Data represent the mean \pm s.e.mean of fourteen to eighteen observations. *** P <0.001 indicates a significant difference from control.

4.2 Time course of ascorbate-induced reversal of acetylcholine-induced vasodilatation to vasoconstriction

The effect of ascorbate on acetylcholine-induced, EDHF-mediated vasodilator responses was not immediate and was only seen when eyes had been perfused from the outset with ascorbate. Therefore, the time course of the ability of ascorbate to inhibit acetylcholine-induced vasodilatation and uncover a vasoconstrictor response was studied over a 120 min period. In control experiments, acetylcholine (10 nmol) induced only vasodilatation and these responses, elicited every 15 min over the 120 min period remained stable (fall in perfusion pressure of $57.6 \pm 6.4 \%$, $n = 6$; Figure 4.3a, 4.4a). In contrast, inclusion of ascorbate (50 μM) into the perfusion medium led to a time-dependent attenuation of the acetylcholine (10 nmol) induced vasodilatation and development of vasoconstriction; vasodilator responses first began to decline after 30 min and were virtually abolished (fall in perfusion pressure of $5.8 \pm 3.0 \%$, $n = 6$) after 120 min (Figure 4.3b, 4.4a). Vasoconstrictor responses (Figure 4.4b), first observed 60 min after treatment with ascorbate (4.3b, 4.4b), were fully developed and remained stable after 90 min (rise in perfusion pressure of $88.1 \pm 13.7 \text{ mmHg}$, $n = 6$; Figure 4.3b, 4.4b). Once the effects of ascorbate were established, washing out the antioxidant for up to 120 min failed to restore the vasodilator responses to acetylcholine (fall in perfusion pressure of $25.7 \pm 3.8 \%$ $n = 11$; Figure 4.5a). Washing out ascorbate appeared to reduce the acetylcholine-induced vasoconstrictor response (rise in perfusion pressure of $43.6 \pm 21.0 \text{ mmHg}$ $n = 11$; Figure 4.5b) but this fall in the vasoconstrictor response was not statistically significant; furthermore, an actual reduction of the vasoconstrictor response was only seen in 3 of 11 eyes. Therefore the ability of ascorbate to reverse

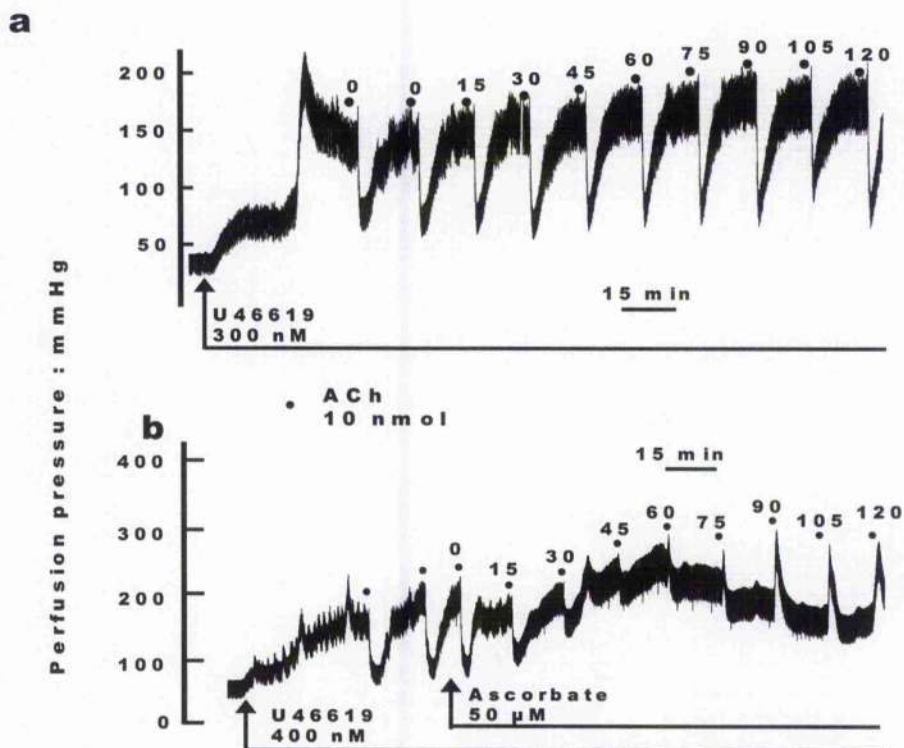


Figure 4.3 Original traces showing acetylcholine-induced responses during time course experiments. (a) Acetylcholine-induced, EDHF-mediated vasodilator responses, elicited every 15 min over a 120 min study period, remained stable. (b) In eyes perfused with ascorbate (50 μ M, >120 min), acetylcholine-induced, EDHF-mediated vasodilatation was blocked in a time-dependent manner and a normally masked vasoconstrictor response to acetylcholine developed. • 0 are control, responses to acetylcholine (10 nmol) to ensure that the vasodilator response was reproducible before starting the time-course experiment or addition of ascorbate.

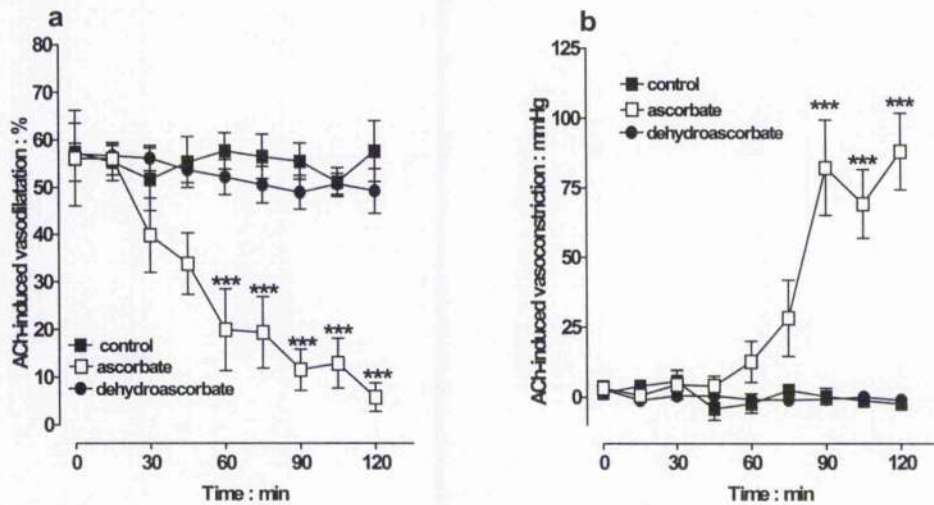


Figure 4.4 Control experiments showing that vasodilator responses to acetylcholine (ACh; 10 nmol) elicited in the bovine isolated perfused eye every 15 min remained stable for a 120 min study period. In contrast, inclusion of ascorbate (50 μ M) in the perfusion medium led to a time dependent loss of vasodilatation (a) and development of vasoconstriction (b). The redox-inactive analogue, dehydroascorbate (50 μ M) lacked the activity of ascorbate. Data represent the mean \pm s.e.mean of six to seven observations. *** $P < 0.001$ indicates a difference from control

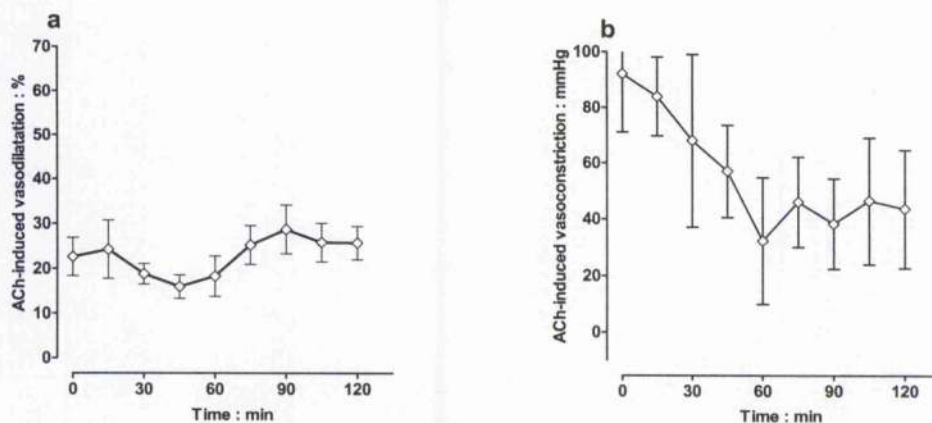


Figure 4.5 In eyes perfused with ascorbate (50 μ M, >120) acetylcholine-induced vasodilatation was inhibited and a normally suppressed vasoconstrictor response developed. These graphs show the results experiments conducted to see if washing out ascorbate over a 120 min period would reverse these effects, i.e. restore acetylcholine-induced vasodilator responses (a) or attenuate vasoconstriction (b). Data represent the mean \pm s.e.mean of eleven observations.

acetylcholine-induced, EDHF-mediated vasodilatation to vasoconstriction is slow and the effect cannot be quickly washed out.

4.3 Concentration-dependence of the ability of ascorbate to reverse acetylcholine-induced vasodilatation to vasoconstriction

The concentration of ascorbate (50 μM) used in experiments was chosen as it is close to the normal plasma concentration, therefore experiments were conducted to assess if ascorbate could inhibit acetylcholine-induced, EDHF-mediated vasodilatation to vasoconstriction across the entire physiological range of blood plasma concentrations. In these experiments bovine eyes were perfused with a range of concentrations of ascorbate (10, 50 and 150 μM) for 120 min before constructing dose-response curves to acetylcholine (1 pmol-1 μmol). Figure 4.6 shows that the ability of ascorbate to inhibit the acetylcholine-induced vasodilator response and uncover a vasoconstrictor response was indeed concentration-dependent, with the highest concentration (150 μM) causing the largest degree of blockade (Figure 4.6). Nevertheless, even the lowest concentration of ascorbate tested (10 μM) resulted in a significant loss of acetylcholine-induced vasodilatation and development of vasoconstriction (Figure 4.6). Therefore, it would appear ascorbate can inhibit acetylcholine-induced, EDHF-mediated vasodilatation in a concentration-dependent manner, across the normal physiological range.

4.4 Time course of ascorbate-induced blockade of bradykinin-induced vasodilatation

Having established that acetylcholine-induced EDHF-mediated vasodilator responses were attenuated by ascorbate, experiments were conducted to determine if bradykinin-induced, EDHF-dependent vasodilator responses were also blocked by ascorbate in the

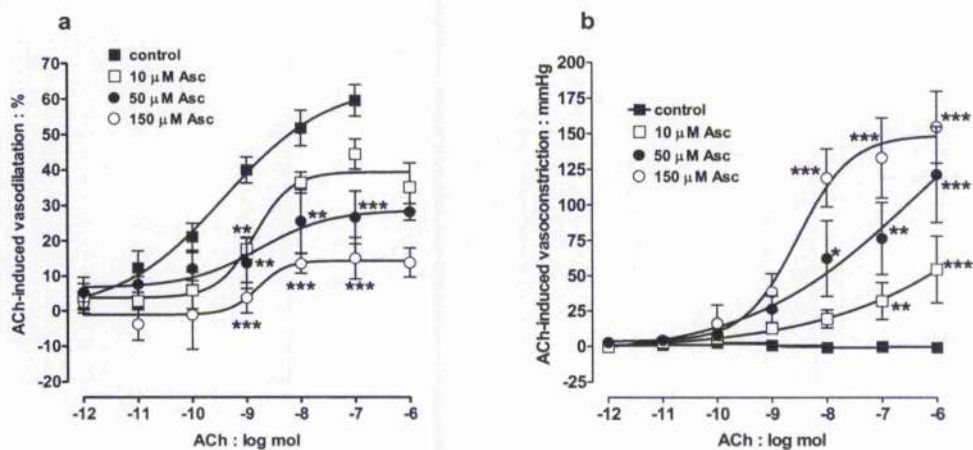


Figure 4.6 Dose-response curves showing acetylcholine (ACh; 1 pmol-1 μmol)-induced responses in the bovine isolated perfused eye. Under control conditions, acetylcholine produced only vasodilator responses ($R_{\max} = 65.4 \pm 5.6 \%$, $pED_{50} = 9.4 \pm 0.2$.) In contrast, following perfusion for 120 min with ascorbate (Asc; 10, 50 or 150 μM), a concentration-dependent loss of vasodilatation (a) and the development of vasoconstriction (b) was seen. Responses in the presence of ascorbate were therefore biphasic, consisting of an initial vasoconstriction followed by a residual vasodilatation. In the presence of 10 μM ascorbate: $R_{\max} = 40.1 \pm 3.0 \%$ $pED_{50} = 8.8 \pm 0.2$ and a vasoconstriction of 59.4 mmHg at 1 μmol ACh; in the presence of 50 μM ascorbate: $R_{\max} = 29.0 \pm 2.7 \%$, $pED_{50} = 8.9 \pm 0.4$ and a vasoconstriction of 155.3 ± 43.8 mmHg at 1 μmol ACh; in the presence of 150 μM ascorbate: $R_{\max} = 14.3 \pm 1.7\%$, $pED_{50} = 8.8 \pm 0.3$ and maximum vasoconstriction= 237.1 ± 13.7 mmHg, $pED_{50} = 8.1 \pm 0.1$. Data represent the mean ± s.e.mean of 5-10 observations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate a difference from control.

bovine eye. In control experiments bradykinin (10 pmol) induced vasodilator responses ($43.8 \pm 3.9 \%$, $n = 8$), and these, elicited every 15 min, remained stable over the 120 min study period (Figure 4.7). In contrast, inclusion of ascorbate into the perfusion medium led to a time-dependent attenuation of bradykinin-induced vasodilatation; vasodilator responses began to decline after 60 min and were virtually abolished after 120 min (Figure 4.7). In contrast to acetylcholine, vasoconstrictor responses were never observed, even when the vasodilator blockade by ascorbate was fully established.

4.5 Effects of antioxidants on acetylcholine-induced vasodilatation

One of the main functions of ascorbate in the body is as an antioxidant; therefore experiments were conducted to assess if the ascorbate-induced blockade of EDHF-mediated vasodilatation was due to an antioxidant action. In these experiments, control vasodilator responses to acetylcholine (10 nmol) were obtained before examining the effects of a range of agents over the ensuing 120 min. Inclusion of the redox-inactive analogue, dehydroascorbate (50 μ M) in the perfusion medium failed to reproduce the reversal of acetylcholine-induced vasodilatation to vasoconstriction seen with ascorbate (Figure 4.4). Moreover, superoxide dismutase (250 units ml^{-1}) and catalase (1250 units ml^{-1}), which scavenge superoxide anion and hydrogen peroxide, respectively, also had no effect on acetylcholine-induced vasodilatation (Figure 4.8).

Perfusion with the endogenous antioxidant and thiol reducing agent, glutathione (1 mM) also had no effect on acetylcholine-induced vasodilatation (Figure 4.8). In contrast, perfusion with two other thiol-reducing agents, N-acetyl-L-cysteine (1mM) and dithiothreitol (100 μ M), did inhibit vasodilatation and uncovered a vasoconstrictor

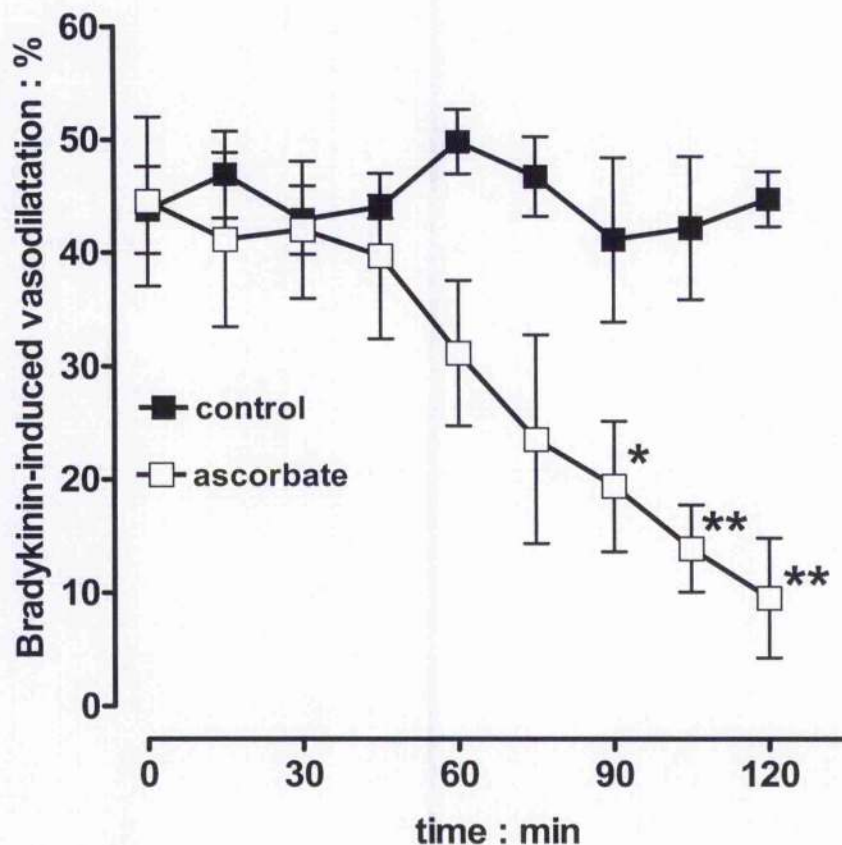


Figure 4.7 Control time course experiments showing that vasodilator responses to bradykinin (10 pmol), elicited in the bovine isolated perfused eye every 15 min, remained stable for a 120 min study period. In contrast, inclusion of ascorbate (50 μ M) in the perfusion medium led to a time-dependent loss of vasodilatation. Data represent the mean \pm s.e.mean of five to nine observations. * $P < 0.05$ and ** $P < 0.01$ indicate a difference from control.

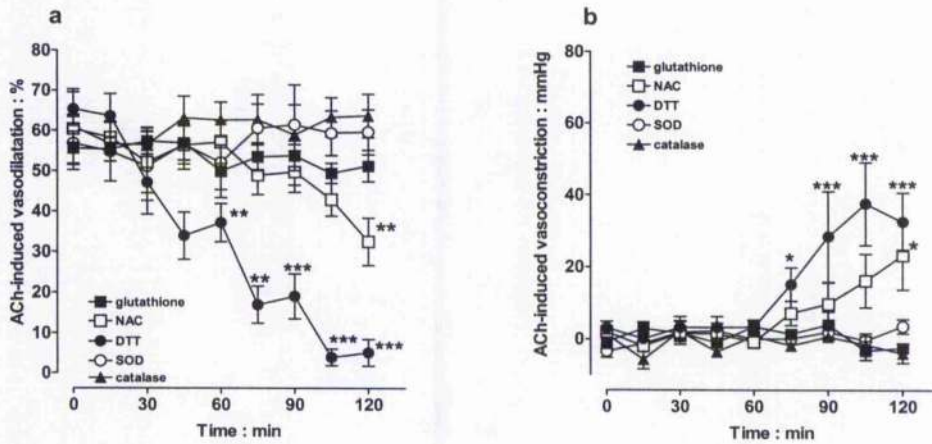


Figure 4.8 After having obtained control vasodilator responses to acetylcholine (ACh; 10 nmol), the effects of infusion of a number of antioxidants, namely, glutathione (1 mM), N-acetyl-L-cysteine (NAC; 1 mM), dithiothreitol (DTT; 100 μ M), superoxide dismutase (SOD; 250 units ml^{-1}) and catalase (1250 units ml^{-1}), were examined on these vasodilator responses over the ensuing 120 min. Loss of acetylcholine-induced vasodilatation and any development of vasoconstriction are shown in a and b, respectively. Data represent the mean \pm s.e.mean of five to ten observations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate a difference from control.

response to acetylcholine, with dithiothreitol being the most effective (Figure 4.8). Thus, the ascorbate-induced blockade of EDHF-mediated vasodilatation can be mimicked by other antioxidants and may involve the reduction of a critical disulphide group.

4.6 Selectivity of the blockade of EDHF-mediated vasodilatation by ascorbate in the ciliary vascular bed of the bovine eye

It is possible that ascorbate could non-selectively attenuate vasodilator responses in the bovine eye. Therefore, experiments were conducted to determine if vasodilator responses mediated by mechanisms other than EDHF could be inhibited by ascorbate. The vasodilator responses to 10 nmol of the nitric oxide donor, glyceryl trinitrate were completely unaffected following treatment with ascorbate (50 μ M, >120 min; Figure 4.9a). Similarly, dose-dependent vasodilatations induced by the opener of K_{ATP} channels, levcromakalim (100 pmol – 30 nmol) were unaffected in the presence of ascorbate (Figure 4.9b). Furthermore the vasoconstrictor response induced by the nitric oxide synthase inhibitor, L-NAME (100 μ M) was unaffected in the presence of ascorbate (increase in perfusion pressure of 30.0 ± 6.0 and 30.5 ± 5.9 mmHg, respectively). These results strongly support the hypothesis that ascorbate selectively attenuates EDHF-mediated vasodilatation in the bovine eye.

4.7 Effects of ascorbate on acetylcholine-induced, EDHF-mediated vasodilatation in the rat isolated perfused mesenteric arterial bed

Experiments were conducted to determine if the ability of ascorbate to attenuate EDHF-dependent vasodilator responses in the bovine eye could also be observed in a preparation where an EDHF-mediated vasodilator response had been previously

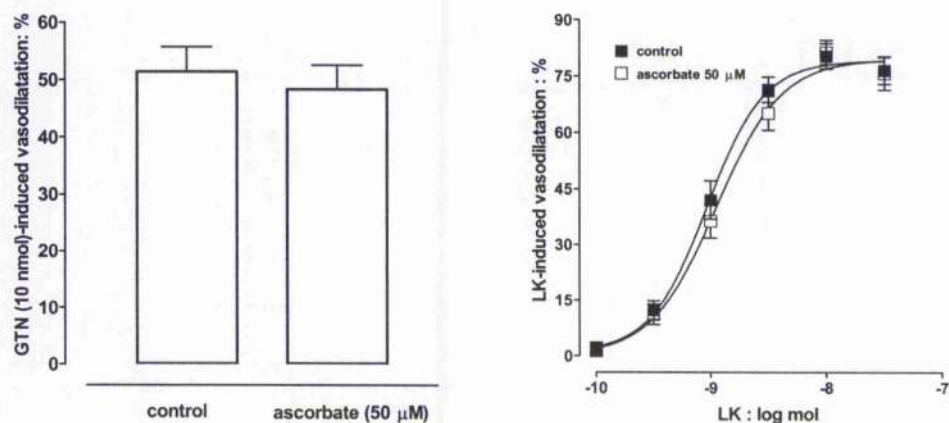


Figure 4.9 (a) Histogram showing the effect of infusing ascorbate (50 μ M, >120 min) on vasodilations induced by the nitric oxide donor, glyceryl trinitrate (GTN, 10 nmol). (b) Dose-response curve showing the effect of infusing ascorbate (50 μ M, >120 min) on vasodilations induced by the opener of K_{ATP} channels, levcromakalim (LK, control $R_{max} = 79.1 \pm 2.1$, $pED_{50} = 9.0 \pm 0.4$, in presence of ascorbate $R_{max} = 79.5 \pm 3.7$, $pED_{50} = 9.0 \pm 0.1$; 0.1 – 30 nmol). Data represent the mean \pm s.e.mean of six to seven observations.

characterised, the rat perfused mesenteric arterial bed preparation (Adeagbo & Triggle, 1993; McCulloch *et al.*, 1997). Following perfusion with Krebs solution and vasoconstriction with phenylephrine (3-10 μ M) to give a final perfusion pressure of 102.3 ± 6.0 mmHg ($n = 6$), acetylcholine (1 pmol-100 nmol) elicited vasodilatations that were dose-dependent (Figure 4.10a). The EDHF-dependent component of vasodilatation was revealed by inclusion of the nitric oxide synthase inhibitor, L-NAME (100 μ M), and the cyclo-oxygenase inhibitor, indomethacin (10 μ M), in the perfusion medium (McCulloch *et al.*, 1997). This EDHF-component of acetylcholine-induced vasodilatation was inhibited when ascorbate (50 μ M) was present in the perfusion medium for 180 min (Figure 4.10a). In contrast to the bovine eye, vasoconstrictor responses to acetylcholine were never observed in the rat mesentery even when vasodilator blockade by ascorbate was fully established.

Experiments were conducted to determine the time course over which ascorbate (50 μ M) attenuated acetylcholine-induced vasodilatation in the rat mesentery. In control experiments acetylcholine (10 nmol)-induced vasodilatation, elicited every 15 min, did not change significantly over a 180 min study period (Figure 4.10b). In contrast, inclusion of ascorbate (50 μ M) in the perfusion medium lead to a time-dependent attenuation of acetylcholine-induced vasodilatation: vasodilator responses first began to decline at 120 min and fell steadily during the remaining 60 min (Figure 4.10b). These results indicate that ascorbate can inhibit EDHF-mediated vasodilatation in vascular beds other than the ciliary vascular bed of the bovine eye.

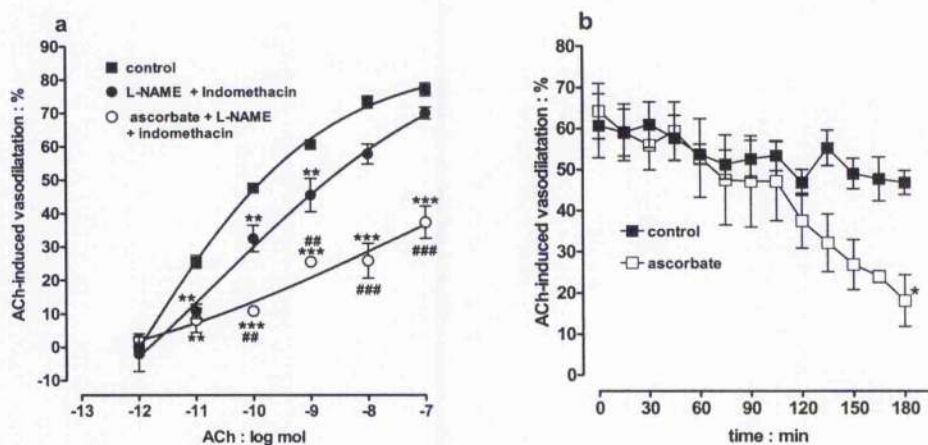


Figure 4.10 Graphs showing the ability of ascorbate to inhibit acetylcholine (ACh)-induced, EDHF-dependent vasodilatation in the rat perfused mesenteric arterial bed. (a) Control dose-response curves to acetylcholine ($R_{\max} = 85.3 \pm 5.7\%$, $pED_{50} = 12.3 \pm 0.1$; 1 pmol-100 nmol) and the residual EDHF-dependent vasodilatation seen following treatment with the combination of L-NAME (100 μ M) and indomethacin (10 μ M; $R_{\max} = 86.3 \pm 22.0\%$, $pED_{50} = 10.4 \pm 1.0$). This residual EDHF-dependent vasodilatation was inhibited following inclusion of ascorbate (50 μ M) in the perfusion medium for 180 min (vasodilatation of $37.3 \pm 4.8\%$ at 100 nmol ACh). (b) Control time course experiments showing that the EDHF-dependent vasodilatation produced by acetylcholine (10 nmol) in preparations treated with L-NAME and indomethacin did not change significantly during a 180 min study period. In contrast, inclusion of ascorbate (50 μ M) into the perfusion medium led to a time-dependent loss of vasodilatation. Data represent the mean \pm s.e.mean of five to eleven observations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate a difference from control. ## $P < 0.01$ and ### $P < 0.001$ indicate a significant blockade by ascorbate of EDHF-induced vasodilator responses, obtained in the presence of L-NAME and indomethacin.

4.8 Effects of ascorbate on bradykinin-induced, EDHF-mediated vasodilatation in the porcine left anterior descending coronary artery. N.B. These experiments were conducted by a colleague, Silvia Nelli

The ability of ascorbate to attenuate EDHF-mediated vasodilatation in the bovine eye and rat mesentery may be due to the small resistance arterioles which control the perfusion pressure; therefore experiments were conducted to assess if ascorbate could attenuate the well characterised EDHF-mediated vasodilator response in the large conduit artery, the porcine coronary artery. In the porcine coronary artery, bradykinin-induced, EDHF-mediated vasodilatation was revealed by inclusion of the nitric oxide synthase inhibitor, L-NAME (100 μ M), and the cyclooxygenase inhibitor, indomethacin (3 μ M), in the bathing medium. In rings bathed in normal Krebs solution the EDHF component of bradykinin (100 pM – 300 nM)-induced vasodilatation was large and concentration-dependent (max 80.3 ± 6.5 %, $n = 10$, Figure 4.11a). In the presence of the BK_{Ca}/IK_{Ca} inhibitor, charybdotoxin (100 nM), the bradykinin-induced, EDHF-mediated vasodilatation was unaffected (maximum vasodilatation of 71.3 ± 9.2 %, $n = 8$, Figure 4.11a). The SK_{Ca} inhibitor, apamin (100 nM), appeared to slightly attenuate the bradykinin-induced EDHF-mediated vasodilatation (maximum vasodilatation of 62.2 ± 5.2 %, $n = 8$, Figure 4.11a), but this was not statistically significant. In contrast, the combination of charybdotoxin plus apamin virtually abolished EDHF-mediated vasodilatation (maximum vasodilatation of 11.3 ± 3.3 %, $n = 10$, $P < 0.001$, Figure 4.11a).

Experiments were conducted to assess the effect of ascorbate on bradykinin-induced, EDHF-mediated vasodilatation in the porcine coronary artery. In these experiments the control vasodilations were completely unaffected (maximum vasodilatation of $76.7 \pm$

4.7 %, $n = 11$, Figure 4.11b) by ascorbate (150 μM , >120 min). Ascorbate also failed to affect bradykinin-induced, EDHF-mediated vasodilatation in the presence of charybdotoxin (maximum vasodilatation of $76.1 \pm 5.9\%$, $n = 8$, Figure 4.11b). Furthermore, ascorbate failed to enhance the small blockade of EDHF-dependent vasodilatation produced by apamin. Thus, ascorbate does not attenuate EDHF-mediated vasodilatation in the porcine coronary artery.

4.9 Effect of inhibiting the ability of the eye to concentrate ascorbate on the blockade of EDHF-mediated vasodilatation in the ciliary vascular bed of the bovine eye

The bovine eye has the ability to concentrate ascorbate in the aqueous humour; therefore experiments were conducted to assess if accumulation of ascorbate in the aqueous humour is a prerequisite for the blockade of EDHF-mediated vasodilatation. The concentration of ascorbate in the aqueous and vitreous humours of unperfused bovine eyes measured within 1 hour of slaughter were 1.1 ± 0.1 and 0.9 ± 0.1 mM ($n = 8$; Figure 4.12), respectively. These concentrations of ascorbate did not change when the eyes were perfused for 120 min with normal Krebs solution (0.9 ± 0.2 and 1.1 ± 0.1 mM, $n = 9$, respectively); perfusion with Krebs solution containing ascorbate (50 μM) also failed to significantly change the concentration of ascorbate in the aqueous and vitreous humours (1.0 ± 0.1 and 0.8 ± 0.1 mM, respectively, $n = 6$; Figure 4.12).

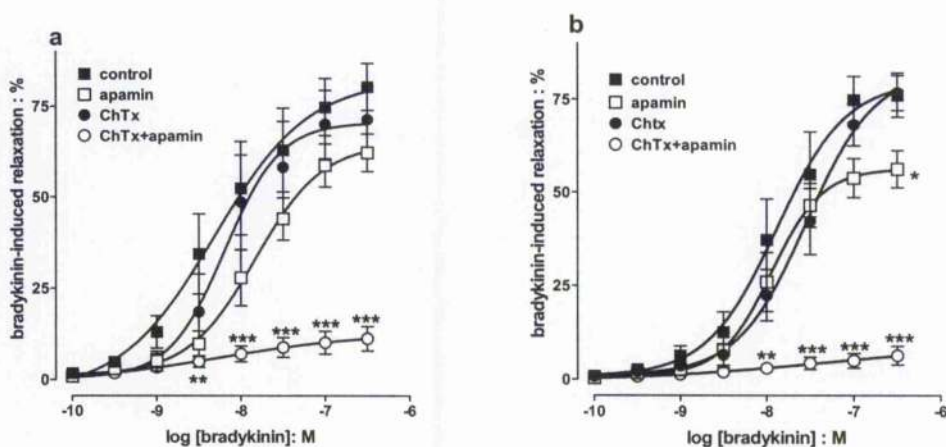


Figure 4.11 Concentration-response curves showing bradykinin (0.1–300 nM; R_{\max} = 82.4 ± 5.7 and 82.9 ± 3.3 %, pED_{50} = 8.3 ± 0.1 and 7.5 ± 0.05 in the absence and presence of ascorbate, respectively)-induced, EDHF-mediated vasodilatation in rings of porcine left anterior descending coronary artery. These also show the effects of the inhibitor of small conductance calcium-sensitive potassium channels (SK_{Ca}), apamin (100 nM; R_{\max} = 64.9 ± 2.0 and 56.3 ± 0.5 %, pED_{50} = 7.8 ± 0.04 and 7.9 ± 0.01 , in the absence and presence of ascorbate, respectively), and of the intermediate conductance calcium-sensitive potassium channels (IK_{Ca}), charybdotoxin (ChTx, 100 nM; R_{\max} = 70.5 ± 2.6 and 79.8 ± 3.6 %, pED_{50} = 8.2 ± 0.1 and 7.9 ± 0.07 , in the absence and presence of ascorbate respectively), each alone and in combination (R_{\max} = 12.8 ± 0.4 and 10.3 ± 3.4 %, pED_{50} = 8.2 ± 0.04 and 7.2 ± 0.6 , in the absence and presence of ascorbate, respectively). Experiments were conducted in the absence (a) and presence (b) of ascorbate (150 μ M). All responses were obtained in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μ M), and the cyclo-oxygenase inhibitor, indomethacin (3 μ M). Data represent the mean \pm s.e.mean of eight to eleven observations. * P <0.05, ** P <0.01 and *** P <0.001 indicate a difference from control.

In experiments where the anterior and posterior chambers were continually flushed with Krebs solution (0.25 ml min^{-1}) to prevent the accumulation of ascorbate (see Figure 2.2) the concentration in the aqueous humour was below detectable limits ($<10 \text{ } \mu\text{M}$, Figure 4.12); however, flushing had no effect on the concentration of ascorbate in the vitreous humour, even when the eyes were perfused with Krebs containing ascorbate (1.0 ± 0.06 and $0.7 \pm 0.08 \text{ mM}$, respectively, $n = 6$). The process of flushing the anterior and posterior chamber of the eye had no effect on control acetylcholine-induced, EDHF-mediated vasodilatation obtained over a 120 min period when eyes were perfused with Krebs solution lacking ascorbate (Figure 4.13). Flushing also failed to prevent the blockade of acetylcholine-induced EDHF-mediated vasodilatation and the uncovering of a vasoconstrictor response when eyes were perfused with Krebs solution containing ascorbate ($50 \text{ } \mu\text{M}$, $>120 \text{ min}$, Figure 4.13).

In some experiments the aqueous humour and vitreous humours of the eye were removed in order to create a preparation with no intact ocular chambers or "reservoirs" of ascorbate. When the ciliary vascular bed was perfused after removal of both the aqueous and vitreous humours the EDHF-mediated vasodilatation was still blocked when ascorbate ($50 \text{ } \mu\text{M}$, $>120 \text{ min}$) was included in the perfusion medium (Figure 4.13). These experiments indicate that ability of ascorbate to attenuate EDHF-mediated vasodilatation in the bovine eye is a direct vascular effect and does not involve the eye's ability to concentrate ascorbate in the aqueous and vitreous humours.

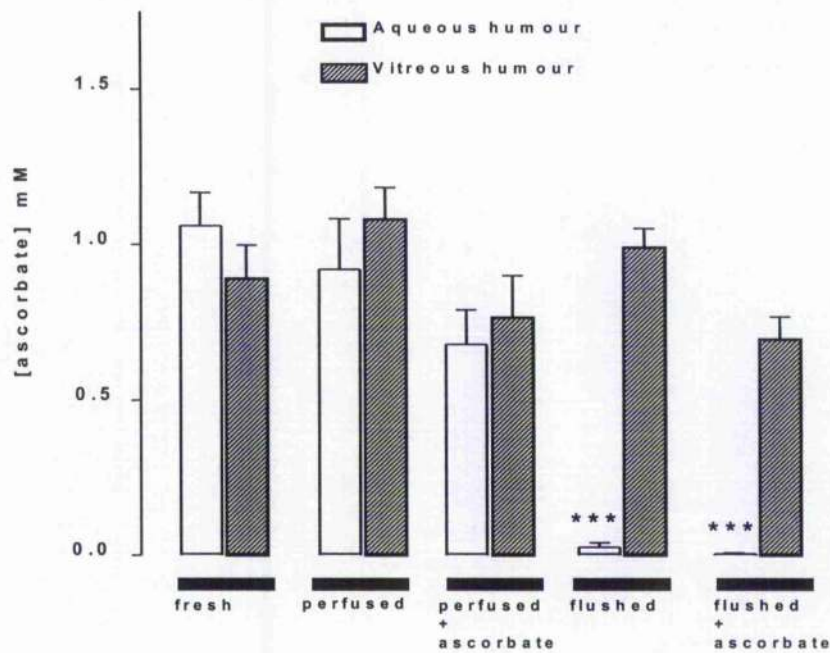


Figure 4.12 Histogram showing the concentration of ascorbate found in samples of aqueous humour and vitreous humour taken from freshly obtained bovine eyes (fresh), from eyes perfused via a ciliary artery for 120 min with normal Krebs solution (perfused) or with Krebs solution containing ascorbate (perfused + ascorbate, 50 μ M). Also shown are the levels of ascorbate found following continuous flushing of the anterior chamber with Krebs solution in eyes whose ciliary vascular bed had been perfused with either normal Krebs solution (flushed) or Krebs containing ascorbate (flushed + ascorbate, 50 μ M). Data represent the mean \pm s.e.mean of six to nine observations. *** $P < 0.001$ indicates a significant difference from the level of ascorbate in fresh eyes.

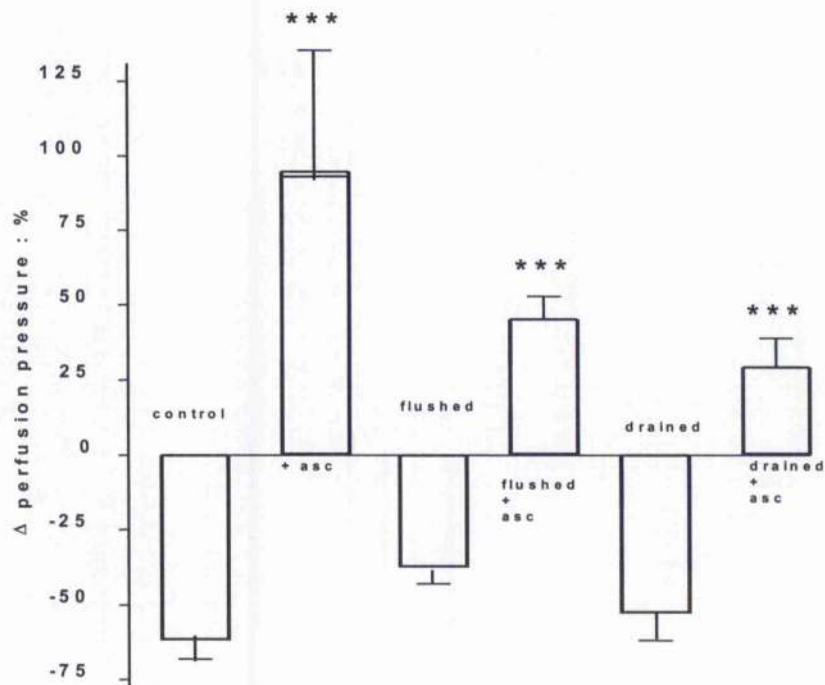


Figure 4.13 Histogram showing control acetylcholine (10 nmol)-induced, EDHF-mediated vasodilatation in the ciliary vascular bed of the bovine perfused eye. This response was blocked, and a normally suppressed vasoconstrictor response uncovered, following treatment with ascorbate (+ asc, 50 μ M, >120 min). When the anterior chamber of the eye was continuously flushed with Krebs solution to prevent the accumulation of ascorbate in the aqueous humour (flushed), or when the aqueous humour and vitreous humour were removed (drained), normal vasodilator responses to acetylcholine were seen. Nevertheless, when ascorbate (50 μ M, >120 min) was added to the perfusion fluid under these conditions, it still resulted in blockade of the EDHF-mediated vasodilatation (flushed + asc, drained + asc, respectively). Data represent the mean \pm s.e.mean of five to eight observations. *** $P < 0.001$ indicates a significant blockade of the EDHF-mediated vasodilatation by ascorbate.

Chapter 5

Discussion



5.1 Perfusion of the ciliary vascular bed of the bovine eye and responses to vasoconstrictor agonists

The main aim of this study was to investigate endothelium-dependent vasodilatation in the ciliary vascular bed of the bovine eye. However, before characterising the vasodilator responses in this tissue it was important to observe the behaviour of this vascular bed under basal, unstimulated perfusion conditions so that the correct conditions could be selected to examine vasodilator responses. When bovine eyes were perfused at a constant flow rate of 2.5 ml min^{-1} the basal perfusion pressure was $38 \pm 1.5 \text{ mmHg}$ ($n = 105$). Experiments were conducted to determine if this perfusion pressure was due simply to the resistance to flow in the preparation, or if it was due to intrinsic (myogenic) tone. In order to test the latter possibility a large vasodilator papaverine ($150 \text{ }\mu\text{M}$, a non-selective inhibitor of phosphodiesterases) was infused; papaverine caused a small but significant vasodilator response indicating that there is a very low level of myogenic tone under basal conditions in this preparation. These results are in contrast to those obtained from resistance vessels from many vascular beds including the mesenteric and coronary beds (Miller *et al.*, 1997; Rajagopalan *et al.*, 1995; Sun *et al.*, 1992) where myogenic tone is observed.

Inhibition of nitric oxide synthase has been shown to cause elevations in blood pressure *in vivo* (Rees *et al.*, 1990), and *in vitro* (Mügge *et al.*, 1991) and inhibiting guanylate cyclase increases resting tone in isolated vessels such as the rabbit aorta (Martin *et al.*, 1986); these observations indicate that, in some vascular preparations, there may be a basal release of nitric oxide (for review see; Martin, 1988). Therefore, experiments were conducted to establish if basal release of nitric oxide was suppressing basal perfusion pressure in the ciliary vascular bed of the bovine eye. In these experiments,

inhibition of nitric oxide synthase with L-NAME (100 μ M) had no effect on the basal perfusion pressure. However, inhibition of soluble guanylate cyclase with ODQ (10 μ M; Garthwaite *et al.*, 1995), resulted in a small vasoconstrictor response indicating that despite the lack of effect of L-NAME, there is probably a small basal production of nitric oxide under resting perfusion conditions. This conclusion was based on the assumption that ODQ is more effective at terminating the actions of nitric oxide than L-NAME, and that the ODQ-induced vasoconstrictor response is not due to a non-selective action of this agent. However, it would be easier to conclude whether there was a basal release of nitric oxide if the myogenic tone was larger; results obtained in the presence of vasoconstrictor agonists appear to confirm the conclusion that there is a basal release of nitric oxide (see below). Nevertheless, these results indicate that there is a small component of intrinsic tone, which may be suppressed by a tonic influence of nitric oxide in the ciliary vascular bed of the bovine eye.

In order to observe vasodilator responses the perfusion pressure must first be increased using a vasoconstrictor agonist. Five commonly used vasoconstrictor agonists were examined in the ciliary vascular bed of the bovine eye in order to assess their suitability: prostaglandin $F_{2\alpha}$; the thromboxane A_2 -mimetic, U46619; the non-selective adrenoceptor agonist, noradrenaline; the selective α_1 -adrenoceptor agonist, phenylephrine; and 5-HT. All these drugs caused concentration-dependent rises in perfusion pressure with the rank order of potency: 5-HT > U46619 > noradrenaline > prostaglandin $F_{2\alpha}$ > phenylephrine. However, due to problems with poor solubility, full concentration-response curves to prostaglandin $F_{2\alpha}$ and phenylephrine could not be obtained, even though these agents produced vasoconstrictor responses. Therefore these drugs were not used in further experiments.

Having established that concentration-dependent vasoconstriction was obtainable, experiments were conducted to determine which of the remaining vasoconstrictor agonists would be most suitable for use in experiments where vasodilator responses were to be elicited. In these experiments, the effect of inhibiting nitric oxide synthase was assessed following precontraction with submaximal concentrations of U46619, 5-HT or noradrenaline. Inhibition of nitric oxide synthase with L-NAME (100 μ M) resulted in a further increase in perfusion pressure of around 30 mmHg, regardless of the vasoconstrictor used. These results suggest that when the perfusion pressure is elevated with an agonist, the basal release of nitric oxide (alluded to above) is exerting a considerable tonic vasodilator effect in the resistance vessels of the bovine ciliary vasculature. Indeed, under similar conditions, a tonic inhibitory action of nitric oxide has been reported in the porcine isolated perfused eye preparation (Meyer *et al.*, 1993). Whether this "basal" release of nitric oxide results from a tonic unstimulated release (Martin *et al.*, 1986), from release stimulated by flow-induced shear stress (Rubyani *et al.*, 1986), from release due to stimulation of the vascular endothelium by the vasoconstrictor agonists themselves (Cocks & Angus, 1983), or even release from nitrergic nerves (Toda & Okamura, 1990), remains to be determined. Nevertheless, studies using bovine isolated retinal arterial rings (Benedito *et al.*, 1991) and porcine ophthalmic and ciliary arterial rings (Haefliger *et al.*, 1993) in tissue baths where shear stress is absent also indicate the presence of a tonic vasodilator action of basal nitric oxide. Moreover, the ability of inhibitors of nitric oxide synthase to reduce ocular blood flow in species as diverse as rat (Koss, 1998), rabbit (Nilsson, 1996), dog (Kitamura *et al.*, 1993) and human (Luksch *et al.*, 2000), demonstrates an important role for basal nitric oxide release in regulating blood flow in the eye.

Experiments were then conducted to determine if vasodilator responses could be elicited in the presence of vasoconstrictor agonists. In these, the ciliary vascular bed was again precontracted (to ~120 mmHg) with submaximal concentrations of noradrenaline, 5-HT, or U46619 and the effect of the commonly used endothelium-dependent vasodilator, acetylcholine (1 pmol - 100 nmol; Furchgott & Zawadzki, 1980), investigated. In these experiments, acetylcholine produced dose-dependent vasodilator responses regardless of the agonist used to raise perfusion pressure. However, the vasodilator responses obtained in the presence of U46619 (maximum fall of perfusion pressure of 59.5 ± 4.9 %) were far larger than those observed in the presence of noradrenaline (maximum fall of perfusion pressure 29.0 ± 5.2 %) or 5-HT (maximum fall of perfusion pressure 10.5 ± 4.6 %). A possible reason that the acetylcholine-induced vasodilatation was smaller when 5-HT or noradrenaline were used to increase perfusion pressure, is that these vasoconstrictors are both known to stimulate release of nitric oxide from the vascular endothelium (Cocks & Angus, 1983; Cohen *et al.*, 1983). Therefore, any additional stimulation of the endothelium may not cause vasodilatation, as nitric oxide release may already be close to maximal. Alternatively, the vasodilator response may have been reduced as the antioxidant, ascorbate (50 μ M), was included in the Krebs solution to prevent these drugs from oxidising, whereas the more stable U46619 was infused in Krebs containing no ascorbate. At this stage of the study the action of ascorbate on endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation was unknown and for simply pragmatic reasons U46619 was used to precontract the ciliary vascular bed in all subsequent experiments.

5.2 Acetylcholine- and bradykinin-induced vasodilatation in the bovine eye is endothelium-dependent but resistant to blockade of nitric oxide synthase and cyclo-oxygenase

When U46619 was used as a vasoconstrictor, acetylcholine and bradykinin both produced dose-dependent vasodilatation in the ciliary vascular bed of the bovine eye. Therefore, experiments were conducted to investigate the role of the endothelium in the responses to these commonly used vasodilators. In these experiments, infusion of the detergent, CHAPS, which has previously been used to induce selective damage of the vascular endothelium in the rat perfused mesentery preparation (Randall & Hiley, 1988), almost abolished the vasodilator responses to acetylcholine and bradykinin in the bovine eye. Surprisingly, CHAPS failed to produce the same increase in U46619-induced perfusion pressure seen when basal nitric oxide activity was abolished with L-NAME. However, it is possible that CHAPS might have caused some slight additional damage to the vascular smooth muscle, which obscured this additional rise. Although, as the perfusion pressure obtained before the addition of CHAPS was maintained after infusion of this agent, any damage to the smooth muscle could not have been too great. A recent report suggested that rather than destroy the endothelium, detergents may simply solubilise receptors on the vascular endothelium and that infusing distilled water instead, was a more effective way to remove the endothelium (Ralevic & Randall, 2002). However, in the bovine eye, perfusing with distilled water for up to 5 minutes had no effect on the acetylcholine-induced vasodilator responses (data not shown). Regardless, based on the results with CHAPS in this study, it seems reasonable to conclude that the vasodilator responses to bradykinin and acetylcholine are endothelium-dependent in the ciliary vascular bed of the bovine eye.

Having established that the vasodilatations to acetylcholine and bradykinin were likely to be endothelium-dependent, experiments were conducted to investigate whether they were mediated by nitric oxide. In these experiments, despite nitric oxide being tonically active, vasodilatations were completely unaffected by the inhibition of nitric oxide synthase using L-NAME. Furthermore, the inhibitor of soluble guanylate cyclase, ODQ, also failed to inhibit the vasodilator response to acetylcholine. Thus, these findings indicate that nitric oxide does not contribute at all to the vasodilator responses to acetylcholine or bradykinin in the bovine eye. These findings are surprising and are in sharp contrast to those obtained in isolated rings of ocular arteries such as the porcine ophthalmic artery (Yao *et al.*, 1991) and ciliary artery (Haefliger *et al.*, 1993; Zhu *et al.*, 1997), and strips of monkey ciliary artery (Toda *et al.*, 1998), where vasodilatations to both bradykinin and acetylcholine were sensitive to inhibition of nitric oxide synthase. However, uncharacterised, residual vasodilatations were observed in all these preparations. It is possible that nitric oxide has greater importance in regulating vascular tone in larger vessels than in resistance vessels in the bovine ciliary vasculature. Indeed, such a decreasing role for nitric oxide with decreasing blood vessel diameter has been noted in the rat mesenteric vascular bed (Hwa *et al.*, 1994). However, in direct contrast to the findings in the bovine eye reported here, the bradykinin-induced vasodilator responses in the porcine isolated perfused eye were blocked by L-NAME (Meyer *et al.*, 1993), suggesting an important role for nitric oxide in such responses in this species. Furthermore, the vasodilatation to acetylcholine *in vivo* in the retinal circulation of newborn pigs, is sensitive to inhibition of nitric oxide synthase (Gidday & Zhu, 1995). In the study of the porcine eye by Meyer and colleagues (1993), constant pressure conditions were used and vasodilatation was measured as a change in flow, whereas in this study, a constant flow rate was employed and vasodilatation was measured as a

change in perfusion pressure; it may be possible that these differences in experimental procedure explain the different sensitivity of the vasodilator response to inhibition of nitric oxide synthase. Regardless of the methodology used, it is more likely that the insensitivity of vasodilatation in the bovine eye to inhibition of nitric oxide synthase represents a species difference.

The vasodilator responses to acetylcholine and bradykinin in the bovine eye are also completely unaffected by treatment with the cyclo-oxygenase inhibitor, flurbiprofen (Nozu, 1978), thus indicating that vasodilatation is not mediated by prostacyclin. Furthermore, the vasodilator responses were unaffected following combined treatment with flurbiprofen and L-NAME. It is, therefore, clear that neither prostacyclin nor nitric oxide is responsible for the vasodilator responses to acetylcholine or bradykinin in the ciliary vascular bed of the bovine eye.

The finding that endothelium-dependent vasodilatation induced by acetylcholine or bradykinin is entirely independent of involvement of nitric oxide is somewhat surprising, but similar findings have been made in the rabbit hindlimb (Mügge *et al.*, 1991) and rat renal artery (Jiang & Dusting, 2001), and is highly suggestive that in the bovine eye the vasodilatation is mediated by another process, perhaps EDHF. However, in perfused vascular beds where EDHF-mediated vasodilatation has been observed, such as the rat mesenteric vascular bed (Adeagbo & Triggle, 1993; Randall *et al.*, 1997) and *in vivo* in the human coronary circulation (Kato *et al.*, 1997) or forearm resistance vessels (Honing *et al.*, 2000), it is more common to find it functioning along with nitric oxide. This is also true in isolated strip/ring arterial preparations where EDHF-mediated

responses can be observed, such as in the rat mesenteric or hepatic arteries (Edwards *et al.*, 1998) and porcine coronary artery (Kilpatrick & Cocks, 1994).

5.3 Endothelium-derived hyperpolarizing factor mediates vasodilatation induced by acetylcholine and bradykinin in the ciliary vascular bed of the isolated perfused bovine eye

Having established that the endothelium-dependent vasodilator responses produced by acetylcholine and bradykinin are not mediated by nitric oxide or prostacyclin, experiments were conducted to assess if they were mediated by EDHF. The characterisation of EDHF is complicated as the nature of the EDHF(s) is still unresolved (see Chapter 1). Possible candidates for EDHF include a cytochrome P₄₅₀ metabolite (Bauersachs *et al.*, 1994), perhaps an epoxyeicosatrienoic acid (EET; Fisslthaler *et al.*, 1999), hydrogen peroxide (Matoba *et al.*, 2000), an endogenous cannabinoid (Randall *et al.*, 1996) and potassium ions (Edwards *et al.*, 1998). Moreover, evidence has been reported suggesting that the process of relaxation involves hyperpolarization of endothelial cells (Edwards *et al.*, 1998; Edwards *et al.*, 2000), and possible subsequent gap junctional transmission of this from endothelium to smooth muscle, resulting in smooth muscle relaxation (Chaytor *et al.*, 1998). Regardless of the conflicting evidence for the proposed candidates, there is consensus that EDHF responses are mediated by an increase in K⁺ conductance and are thus sensitive to depolarising solutions of K⁺ (Adcagbo & Triggle, 1993)(the depolarisation is such that the equilibrium potential for K⁺ as predicted by the Nernst equation, will be changed such that opening of K⁺ channels will no longer cause hyperpolarization) and by inhibitors of certain types of K⁺ channels (see below). Consistent with the possibility that EDHF-mediates acetylcholine- and bradykinin-induced vasodilatation in the bovine eye, vasodilatation

was virtually abolished following infusion of a depolarising concentration of K^+ in the Krebs solution (30 mM).

It has been suggested that K^+ released from the endothelium can act as an EDHF by stimulating the Na^+/K^+ ATPase or inwardly rectifying K^+ channels (K^+_{IR}) on smooth muscle of rat hepatic arteries (Edwards *et al.*, 1998). Therefore, the effects of the K^+_{IR} inhibitor, barium, and the Na^+/K^+ ATPase inhibitor, ouabain, were examined on vasodilator responses to acetylcholine in the ciliary vascular bed of the bovine eye. In fact, vasodilatation was unaffected by barium, inhibited by ouabain and further inhibited by the combination of the two inhibitors. The finding that vasodilator responses to acetylcholine in the bovine eye are sensitive to inhibition of the Na/K -ATPase and K_{IR} could be consistent with the hypothesis that K^+ ions can act as EDHF (Edwards *et al.*, 1998). However, the possibility that K^+ released from the endothelium is EDHF has been challenged by the finding that EDHF-mediated responses are unaffected by ouabain or barium in the guinea-pig carotid artery, porcine coronary artery (Quignard *et al.*, 1999) and rat hepatic artery (Andersson *et al.*, 2000). Moreover, in rat mesenteric (Harris *et al.*, 2000; Lacy *et al.*, 2000) and renal arterics (Jiang & Dusting, 2001), potassium-induced vasodilatation is abolished following removal of the endothelium, thus casting doubt on a role for this ion as EDHF in these vessels. However, some of these studies used tension recording to measure the EDHF response and as such required the use of vasoconstrictor agents. Recently it has been demonstrated that the ability of K^+ to mimic EDHF, i.e. to produce hyperpolarization and vasodilatation, decreases as vasoconstrictor tone is increased (Dora & Garland, 2001; Richards *et al.*, 2001). It is thought that the rise in smooth muscle intracellular Ca^{2+} due to presence of vasoconstrictors can open K_{Ca} channels, causing K^+ release from smooth muscle cells

creating an extracellular K^+ "cloud". This "cloud" would maximally activate the Na/K-ATPase and K_{IR} such that it would prevent hyperpolarisation of smooth muscle cells upon release of more K^+ (EDHF?) from the endothelial cells (Dora & Garland, 2001). These findings indicate that in experiments employing vasoconstrictor agonists in order to measure decreases in tension (or perfusion pressure) to vasodilators, care must be taken in interpreting the role of K^+ in the EDHF-mediated responses.

Having established that a high K^+ concentration inhibited endothelium-dependent vasodilatation in the bovine eye, the possible involvement of potassium channels was investigated on acetylcholine-induced vasodilatation. K_{ATP} channels are not generally considered to be involved in EDHF-mediated vasodilatation (Corriu *et al.*, 1996) and consistent with this, addition of glibenclamide, a blocker of K_{ATP} channels (Feelisch & Noack, 1987), had no effect on the vasodilatation induced by acetylcholine in the bovine eye. Tetraethylammonium (TEA) is a relatively non-selective inhibitor of calcium-sensitive K^+ (K_{Ca}) channels (Cook & Quast, 1990), although it does not block small conductance K_{Ca} (SK_{Ca}), even at concentrations up to 4 mM (Povstyan *et al.*, 2000). Infusion of TEA caused a large, transient vasoconstrictor response, indicating that K_{Ca} channels are probably tonically active in the bovine eye. In addition, the acetylcholine-induced vasodilatation was blocked by ~50% by TEA, which is in keeping with its ability to inhibit EDHF-mediated vasodilatation in human forearm resistance vessels (Honing *et al.*, 2000) and in rat mesenteric arteries (Chen & Cheung, 1997).

The vast majority of evidence suggests that the action of EDHF is normally abolished by the combination of the small conductance K_{Ca} (SK_{Ca}) channel blocker, apamin, with

the large and intermediate conductance K_{Ca} (BK_{Ca}/IK_{Ca}) blocker, charybdotoxin, but not by the combination of apamin with the large conductance K_{Ca} (BK_{Ca}) inhibitor, iberiotoxin (Edwards *et al.*, 1998; Edwards *et al.*, 1999a; Zygmunt & Hogestatt, 1996). On the basis of these findings, the EDHF pathway is thought to involve the opening of SK_{Ca} channels and IK_{Ca} channels but not BK_{Ca} channels, on the endothelial cell (Edwards *et al.*, 1998; Ohashi *et al.*, 1999). In the bovine eye, apamin (100 nM) had no significant effect on U46619-induced perfusion pressure but charybdotoxin (50 nM) and iberiotoxin (50 nM) both caused a large transient, vasoconstrictor response of ~300 mmHg lasting ~15 min, similar to that seen with TEA (see above). These vasoconstrictor responses may have been due to inhibiting BK_{Ca} channels and inhibition of these channels is known to inhibit nitric oxide-induced vasodilatation (Demirel *et al.*, 1994) (Champion & Kadowitz, 1997); it is however unlikely that in the bovine eye these large transient vasoconstrictions reflect inhibition of basal nitric oxide activity, since the nitric oxide synthase inhibitor, L-NAME, produced much smaller rises in perfusion pressure (~30 mmHg) which were maintained for many hours. It is more likely that these findings indicate that BK_{Ca} channels are normally open under the conditions used in the current study, as TEA, charybdotoxin and iberiotoxin all inhibit this channel. Indeed, a similar contraction in response to charybdotoxin has been observed in the saphenous artery of the mouse (Ding *et al.*, 2000) and charybdotoxin and iberiotoxin can augment phenylephrine-induced vasoconstriction in rat mesenteric arteries (Dora *et al.*, 2002). Regardless of any effects on U46619-induced perfusion pressure, apamin (100 nM) and iberiotoxin (50 nM) alone or in combination had no significant effect on the acetylcholine-induced vasodilatation. In contrast, charybdotoxin (50 nM) alone significantly attenuated acetylcholine-induced vasodilatation, and uncovered a vasoconstrictor response (see below). In previous studies (Edwards *et al.*,

1998; Edwards *et al.*, 1999a; Zygmunt & Hogestatt, 1996), charybdotoxin alone did not attenuate acetylcholine-induced vasodilatation, as was seen in the ciliary vascular bed of the bovine eye. This may reflect the requirement of the combination of charybdotoxin and apamin to inhibit EDHF in these preparations, or the absence of the unusual acetylcholine-induced vasoconstriction observed in the bovine eye. A lower concentration of charybdotoxin (10 nM) had no effect on perfusion pressure or vasodilatation. Nevertheless, when this lower concentration was combined with apamin, a significant attenuation of acetylcholine-induced vasodilatation was observed. Thus, the attenuation of the acetylcholine-induced vasodilations with the combination of apamin and charybdotoxin, and the lack of effect of the combination of apamin and iberiotoxin is consistent with the possibility that the vasodilator responses observed in the ciliary vascular bed of the bovine eye involve both SK_{Ca} and IK_{Ca} and may be mediated by EDHF.

In an attempt to elucidate further the nature of the EDHF in the ciliary vascular bed of the bovine eye, the effects of a range of potassium channel openers commonly used to characterise, or mimic the effects of EDHF were assessed. EDHF-mediated vasodilatation is not thought to involve the opening of K_{ATP} channels, however the endothelium-independent opener of K_{ATP} channels, levcromakalim (Katnik & Adams, 1997), elicited large vasodilations, thus demonstrating that the vascular smooth muscle cells of the ciliary vascular bed are capable of vasodilatation to hyperpolarizing stimuli independent of EDHF. The opening of IK_{Ca} channels is thought to be involved in the EDHF-response (Edwards *et al.*, 1998; Edwards *et al.*, 1999a) and 1-EBIO is a putative opener of IK_{Ca} channels (Adeagbo, 1999). Moreover, it has previously been used to demonstrate that the opening of endothelial IK_{Ca} channels and the subsequent

release of K^+ may underpin the actions of EDHF (Edwards *et al.*, 1999a). In the bovine eye 1-EBIO caused a small dose-dependent vasodilatation (maximum fall in perfusion pressure of 30.0 ± 4.8 mmHg). A newly introduced, more potent analogue of this, DCEBIO (Singh *et al.*, 2001), did, however, elicit large dose-dependent vasodilator responses (maximum fall in perfusion pressure of 82.2 ± 6.0 mmHg). These results could be consistent with K^+ released from the endothelium being the EDHF in bovine eye and would support the evidence gained from the blockade of EDHF observed in the presence of ouabain and barium. However, the vasodilator responses induced by DCEBIO were completely unaffected by the inhibitor of IK_{Ca} , charybdotoxin (50 nM), indicating that this agent causes vasodilatation, in the bovine eye, by a mechanism distinct from the opening of IK_{Ca} . Indeed, it has been previously reported in the rat mesenteric artery that 1-EBIO could promote vasodilatation at concentrations less than those required to elicit hyperpolarization and this relaxation was insensitive to charybdotoxin or iberiotoxin (Walker *et al.*, 2001). Consequently, the results with this and similar agents should be treated with caution. The effects of the opener of BK_{Ca} channels, NS1619 (Olesen *et al.*, 1994b), were also assessed, but it could only elicit a small vasodilatation at the highest concentration (100 nmol) studied (fall in perfusion pressure of 19.1 ± 2.4 %, $n = 7$). It is unlikely that this vasodilatation in response to NS1619 was poor due to the absence of BK_{Ca} channels in this preparation, as agents which block this channel such as charybdotoxin, iberiotoxin and TEA all produced vasoconstrictor responses (see above). It is more likely that, because the BK_{Ca} channels appear to be constitutively active in the bovine eye, NS1619 cannot produce much further activation of these channels. These results confirm the established literature that a BK_{Ca} channel is unlikely to be involved in the EDHF-mediated vasodilatation in the bovine eye (see above). The effect of the putative candidate for EDHF, anandamide

(Randall *et al.*, 1996), was also examined in the bovine eye. However, in this study, only the highest dose of anandamide studied (100 nmol) produced a small vasodilator response ($21.0 \pm 1.5 \%$), suggesting that some other mechanism underpins the EDHF-mediated response in the ciliary vascular bed of the bovine eye. No further attempt was made to elucidate the nature of the EDHF in this study.

5.4 Ascorbate inhibits EDHF-mediated vasodilatation and uncovers a vasoconstrictor response in the ciliary vascular bed of the bovine eye

Having established that the vasodilatation induced by acetylcholine and bradykinin are mediated by an EDHF-like substance, the effects of the antioxidant, ascorbate, were examined on these vasodilator responses in the ciliary vascular bed of the bovine eye. The effects of ascorbate were studied as this antioxidant is known to protect nitric oxide-dependent vasodilator responses under conditions of oxidant stress (Dudgeon *et al.*, 1998; Fontana *et al.*, 1999). Furthermore, as ascorbate supplied by the plasma is concentrated in the aqueous humour (to ~ 1 mM), previous studies using the bovine isolated perfused eye have included ascorbate in the perfusion medium (Shahidullah & Wilson, 1999; Wilson *et al.*, 1993). Surprisingly, the present study demonstrated that ascorbate, at concentrations similar to those occurring in normal human plasma (mean 46.8 ± 8 , range 30–150 μ M; Keaney & Vita, 1995; Levine *et al.*, 1996), blocks EDHF-mediated vasodilatation. Specifically, when the ciliary vascular bed was perfused with Krebs solution containing ascorbate (50 μ M, >120 min), acetylcholine induced vasoconstrictor responses that were biphasic with a small, residual, vasodilator response. This is in direct contrast to when the ciliary vascular bed is perfused with normal Krebs solution (i.e. containing no ascorbate), where acetylcholine produces only vasodilator responses that are mediated by EDHF (see Chapter 3 and Section 5.2). This

acetylcholine-induced vasoconstriction was unaffected by the cyclo-oxygenase inhibitor, flurbiprofen (30 μ M), eliminating the involvement of a vasoconstrictor prostanoid. The vasoconstriction was, however, abolished by atropine (100 nM), indicating that it was muscarinic in nature, and ruling out the possibility that the vasoconstriction was due to stimulation of nicotinic acetylcholine receptors on perivascular nerves and the subsequent release of noradrenaline. Accordingly it is likely to result from the well-characterised direct contractile action of acetylcholine on vascular smooth muscle (Furchgott & Zawadzki, 1980). In the absence of ascorbate, when CHAPS was infused to selectively destroy the endothelium, acetylcholine-induced vasoconstrictor responses were not always observed. Therefore it may be possible that the vasoconstrictor response produced by acetylcholine may be endothelium-dependent. Indeed, acetylcholine-induced, endothelium-dependent vasoconstrictor responses have been observed in rat aorta (Franchi-Micheli *et al.*, 2000). However, it is likely that in this study CHAPS also caused some damage of the smooth muscle cells (cf. the vasoconstrictor response induced by inhibiting nitric oxide synthase with L-NAME. A similar vasoconstrictor response would be expected upon removal of the endothelium, but was not observed, indicating a degree of smooth muscle cell damage, see Section 5.2) and this damage may have been enough to prevent the acetylcholine-induced vasoconstrictor response.

As reported in Chapter 3 and Section 5.3 a similar "reversal" of vasodilatation to vasoconstriction is seen in the bovine eye when charybdotoxin was used to attenuate acetylcholine-induced EDHF-mediated vasodilatation. Based on this observation, it is likely that ascorbate blocks the EDHF pathway at some point, thus uncovering a normally masked acetylcholine-induced vasoconstrictor response. Indeed, this

conclusion is strengthened by the results of time course experiments. These showed that although control acetylcholine-induced, EDHF-mediated vasodilator responses were reproducible during a 120 min study period, inclusion of ascorbate (50 μ M) in the perfusion medium led to a time-dependent fall in vasodilatation and development of vasoconstriction. By the end of the 120 min period vasodilatation was almost abolished and vasoconstriction had developed fully, while at intermediate time points, a biphasic response, consisting of an initial vasoconstriction followed by weak residual vasodilatation was observed. Furthermore, before the intermediate biphasic responses were observed, the acetylcholine-induced vasodilator response had already begun to decline.

Further investigation revealed that the ability of ascorbate to reverse acetylcholine-induced vasodilatation to vasoconstriction was concentration-dependent, with even the lowest concentration studied (10 μ M), producing a significant attenuation of vasodilatation and uncovering of the vasoconstrictor response. Moreover, at the highest concentration of ascorbate tested (150 μ M), vasodilator responses were virtually abolished, with acetylcholine now producing almost exclusively vasoconstriction. Thus, the inhibitory action of ascorbate was seen over the full range of concentrations normally found in human plasma (30-150 μ M; Keaney & Vita, 1995) and even at concentrations lower than this.

Bradykinin also elicits EDHF-dependent vasodilatation in the bovine isolated perfused eye (see Chapter 3 and Section 5.3), so it was important to determine if the blocking action of ascorbate extended to this vasodilator. In time-course experiments, control bradykinin-induced, EDHF-mediated vasodilator responses were stable during the 120

min study period when the eye was perfused with standard Krebs solution. Moreover, in common with experiments when acetylcholine was used as the vasodilator, inclusion of ascorbate (50 μ M) in the perfusion medium led to a time-dependent fall in bradykinin-induced vasodilatation, such that at 120 min the response was virtually abolished. In contrast to acetylcholine, vasoconstrictor responses to bradykinin were never observed, even when the blocking action of ascorbate was fully established. This observation therefore strengthens the conclusion that the effect of ascorbate in the bovine eye is to block EDHF-mediated vasodilatation and not to invoke a normally-absent vasoconstrictor response. Moreover, these findings indicate that the blocking action of ascorbate is mediated by an action on an element of the EDHF signal transduction cascade common to both acetylcholine and bradykinin.

5.5 Ascorbate inhibits EDHF in the ciliary vascular bed of the bovine eye by an antioxidant action

One of the main functions of ascorbate in the body is to act as a low molecular weight antioxidant (Halliwell & Gutteridge, 1989). Therefore, time course experiments were conducted to assess the possibility that the ascorbate-induced blockade of EDHF-mediated vasodilatation was due to an antioxidant action. In such experiments, the failure of the redox-inactive analogue, dehydroascorbate (50 μ M), to mimic the effects of ascorbate on acetylcholine-induced vasodilatation supports this view. In addition, due to the instability of dehydroascorbate in aqueous solution (Bogdanski & Bogdanska, 1955), these results eliminate the possibility that the breakdown products of dehydroascorbate, i.e. oxalic and L-threonic acids (Halliwell & Gutteridge, 1989), could account for the ability of ascorbate to block EDHF-mediated vasodilatation.

One of the antioxidant actions of ascorbate is to sequester the superoxide anion (Som *et al.*, 1983). The superoxide anion can spontaneously react with water to produce hydrogen peroxide (Halliwell & Gutteridge, 1989). However, in the bovine eye neither superoxide dismutase (250 units ml^{-1}) nor catalase (1250 units ml^{-1}), enzymes which scavenge superoxide and hydrogen peroxide, respectively, shared the ability of ascorbate to block acetylcholine-induced, EDHF-mediated vasodilatation. Thus, the blocking action of ascorbate cannot be attributed to the scavenging of superoxide anion or hydrogen peroxide. The lack of effect of catalase is of additional significance, as two recent studies suggest that hydrogen peroxide is the EDHF in mouse (Matoba *et al.*, 2000), and human (Matoba *et al.*, 2002) mesenteric arteries. Therefore, the lack of effect of catalase, in this study, suggests that hydrogen peroxide is unlikely to underlie EDHF-mediated vasodilatation in the bovine eye.

Glutathione is another endogenous, low molecular weight antioxidant and thiol-reducing agent (Halliwell & Gutteridge, 1989; Kosower & Kosower, 1978). Consequently, experiments were conducted to determine if this antioxidant could mimic the effects of ascorbate in the bovine eye. In such experiments glutathione (1 mM) failed to block acetylcholine-induced, EDHF-mediated vasodilatation. In contrast, two other more powerful thiol-reducing agents, N-acetyl-L-cysteine (1 mM; Weinander *et al.*, 1994) and dithiothreitol (100 μM ; Karlin & Bartels, 1966), each promoted a time-dependent fall in acetylcholine-induced vasodilatation and uncovered a vasoconstrictor response. These results may simply be explained by the general antioxidant action of the two compounds, but also raise the possibility that they (and ascorbate) might block EDHF-mediated vasodilatation by reducing a critical disulphide group at some point on its signal transduction cascade.

Redox modification of thiol groups has been reported to modulate the gating of IK_{Ca} (Cai & Sauve, 1997), which, together with SK_{Ca} , is generally involved in EDHF-mediated vasodilatation (Edwards et al., 1998; Feletou & Vanhoutte, 1999). If IK_{Ca} is the target for ascorbate and the thiol-reducing agents in the bovine eye it would be expected that they would block this channel. This is supported by the charybdotoxin-induced blockade of EDHF, where a biphasic acetylcholine-induced vasoconstriction is also observed. However, IK_{Ca} channels in bovine aortic endothelial cells are reported to have an increased open probability when treated with thiol-reducing agents (Cai & Sauve, 1997). Thus, ascorbate's target may be distinct from this channel.

An alternative locus for the inhibitory action of ascorbate might be cytochrome P450. It has been suggested that EDHF-dependent vasodilatation may be mediated by a product of a cytochrome P450 enzyme (Bauersachs et al 1994; Fisslthaler *et al.*, 1999). Cytochrome P450s are redox sensitive and ascorbate is known to inhibit their activity (Anderson & Kappas, 1991; Ghosh *et al.*, 1997). It would therefore be of interest to determine if inhibition of cytochrome P450 enzymes underlies the ability of ascorbate to block EDHF-mediated vasodilatation. In addition, gap junctions have been implicated in EDHF-mediated vasodilations (Chaytor *et al.*, 1998). Recently it has been reported that a slow blockade of EDHF-mediated responses is associated with the use of HEPES-based buffers, possibly by the binding of an amino-sulphonate moiety to gap junctions, thus reducing connexin expression (Edwards *et al.*, 2001). Thus raising the possibility that ascorbate may act in a similar manner.

The blockade of EDHF-mediated vasodilatation in the bovine eye by ascorbate at concentrations well within the normal plasma range raises several important issues. For example, if these *in vitro* experiments reflect the behaviour of the ciliary vascular bed *in vivo*, they would suggest that EDHF activity is normally greatly suppressed. Such a situation seems almost inconceivable, since numerous reports describe vasodilator responses attributed to EDHF in living animals (Nishikawa *et al.*, 1999; Parkington *et al.*, 2002; Welsh & Segal, 2000) and humans (Honing *et al.*, 2000; Katz & Krum, 2001). Furthermore, the blocking action of ascorbate on EDHF contrasts markedly with its actions on nitric oxide, where enhancement of vasodilator activity is widely reported. For example, ascorbate is able to restore nitric oxide-dependent vasodilatation following its impairment by oxidant stress in isolated arterial rings (Dudgeon *et al.*, 1998) (Fontana *et al.*, 1999). Moreover, acute treatment of patients with ascorbate has led to restoration of impaired nitric oxide-mediated vasodilatation in essential hypertension (Natali *et al.*, 2000; Taddei *et al.*, 1998b), atherosclerosis (Levine *et al.*, 1996), hypercholesterolaemia (L'ing *et al.*, 1997), insulin-dependent (Timimi *et al.*, 1998) and non-insulin-dependent diabetes (Ting *et al.*, 1996) and chronic heart failure (Ellis *et al.*, 2001; Hornig *et al.*, 1998).

Although highly speculative, it is possible that ascorbate may act *in vivo* as a redox switch to activate EDHF under conditions of oxidant stress. Under such conditions nitric oxide is destroyed by the superoxide anion (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986) and low molecular weight antioxidants such as ascorbate are rapidly consumed (see; Frei, 1994). The depletion of ascorbate could then potentially activate the normally suppressed EDHF-dependent vasodilator mechanism. Indeed, support for this concept comes from a recent report showing that in heart failure, where nitric

oxide-dependent vasodilatation is impaired by oxidant stress in the forearm circulation, EDHF becomes the dominant vasodilator mechanism (Katz & Krum, 2001). Further evidence of a reciprocal interaction between these two vasodilators is suggested by the findings that nitric oxide inactivates EDHF in the porcine coronary circulation both *in vitro* (Bauersachs *et al.*, 1996) and *in vivo* (Nishikawa *et al.*, 2000). Furthermore, it has been suggested that the EDHF-mediated component of vasodilatation becomes fully established in porcine isolated coronary arteries only when the nitric oxide-dependent component is inhibited (Kilpatrick & Cocks, 1994). Whatever the precise interaction between these two important vasodilators, the findings with ascorbate in the present study may have important consequences for cardiovascular pathologies where nitric oxide-mediated vasodilatation is impaired by oxidant stress.

5.6 Selectivity of the ability of ascorbate to inhibit EDHF-mediated vasodilatation in the ciliary vascular bed of the bovine eye

It was possible that the blockade of EDHF mediated vasodilatation in the bovine eye was simply the result of a non-selective damage to endothelial cells. Therefore, experiments were conducted to assess if EDHF-independent vasodilations could be affected by ascorbate (50 μ M). In such experiments, the vasodilatation to the nitric oxide donor, glyceryl trinitrate (Feelisch & Noack, 1987) was completely unaffected by ascorbate suggesting that in the presence of ascorbate the nitric oxide effector pathway remains intact. Furthermore, vasodilatation induced by levcromakalim, which causes vasodilatation by opening of K_{ATP} channels on smooth muscle cells (Katnik & Adams, 1997), leading to hyperpolarization of the smooth muscle, was unaffected by the infusion of ascorbate. EDHF-dependent vasodilations are not thought to involve the opening of these glibenclamide-sensitive K_{ATP} channels (Corriu *et al.*, 1996). Indeed in

the bovine eye, EDHF-mediated vasodilatation is not affected by the infusion of glibenclamide.

Both glyceryl trinitrate and levromakalim elicit endothelium-independent vasodilatation and the lack of effect of ascorbate on these show that the smooth muscle cell relaxation mechanisms are intact. They do not, however, eliminate the possibility that ascorbate-induced blockade of EDHF-mediated vasodilatation results from non-selective damage of endothelial cells. This possibility was ruled out, however, as the vasoconstrictor response resulting from the tonic vasodepressor actions of basal nitric oxide, produced by inhibiting nitric oxide synthase with L-NAME, was completely unaffected by the infusion of ascorbate in the ciliary vascular bed of the bovine perfused eye. Thus in the presence of ascorbate the basal nitric oxide activity is still present, suggesting that the endothelium is functional.

5.7 Ascorbate inhibits EDHF-mediated vasodilatation in the rat isolated mesenteric arterial bed

The ciliary body of mammalian eyes actively transports ascorbate (Millar & Kaufman, 1995), therefore the concentration of ascorbate found in the aqueous humour is normally around 1 mM in a wide range of animal species, including humans (Caprioli, 1992; Halliwell & Gutteridge, 1989; Millar & Kaufman, 1995). Consequently, experiments were conducted to determine if the ability of ascorbate to block EDHF-mediated vasodilatation was peculiar to the eye, or a more general phenomenon. Accordingly, the studies were extended to a different preparation where the role of EDHF has been characterised extensively, i.e. the rat isolated perfused mesenteric arterial bed (McCulloch *et al.*, 1997). In the present study acetylcholine-induced

vasodilations were slightly attenuated in the presence of the nitric oxide synthase inhibitor, L-NAME but the maximum vasodilatation was unaffected, and the additional infusion of indomethacin had no further effect on vasodilator responses. These results are in agreement with previous studies in the rat mesentery (McCulloch *et al.*, 1997). Furthermore, the vast majority of studies indicate that, in rat mesenteric arteries, endothelium-dependent vasodilatation resistant to inhibitors of nitric oxide synthase and cyclo-oxygenase are mediated solely by EDHF (Dora & Garland, 2001; Edwards *et al.*, 1998; McCulloch *et al.*, 1997). The present study revealed that acetylcholine (10 nmol)-induced, EDHF-dependent vasodilator responses in the perfused mesentery were indeed blocked in a time-dependent manner when ascorbate (50 μ M) was included in the perfusion medium. The time required for maximal blockade (180 min) was somewhat longer than in the bovine eye (120 min), but time-matched experiments conducted in the absence of ascorbate showed that acetylcholine-induced vasodilatation was well maintained during this period. In addition, blockade by ascorbate (50 μ M, > 180 min) of EDHF-mediated vasodilations induced by acetylcholine occurred across the entire dose-response range (1 pmol-100 nmol) in this preparation. Thus, the ability of ascorbate to block EDHF-mediated vasodilatation is not limited to the bovine eye and appears to be a more widespread phenomenon.

5.8 Ascorbate does not inhibit EDHF-mediated vasodilatation in isolated rings of porcine coronary artery. A colleague, Silvia Nelli, performed the experimental work in this section, in a collaborative study

As has already been established, ascorbate inhibits EDHF-mediated vasodilator responses not just in the ciliary vascular bed of the bovine eye, but also in the mesenteric arterial bed of the rat. As both these preparations are perfused vascular beds

it is likely that the vessels controlling the perfusion pressure are the small resistance arterioles. Thus raising the possibility that they may be the locus of the ascorbate-induced blockade of EDHF. Consequently, experiments were conducted to determine if blockade of EDHF-mediated vasodilatation by ascorbate might also be observed in a large conduit artery. The porcine coronary artery was chosen for this purpose because EDHF-mediated vasodilatation and hyperpolarization have been extensively studied in this tissue (Beny & Schaad, 2000; Edwards *et al.*, 2000; Edwards *et al.*, 2001; Fisslthaler *et al.*, 1999; Fleming *et al.*, 1999). In the present study, and in agreement with the previous reports, it was found that in the porcine coronary artery, EDHF-mediated vasodilatation was uncovered by inhibiting nitric oxide synthase and cyclooxygenase. Inhibiting SK_{Ca} with apamin, or IK_{Ca} with charybdotoxin, each alone, had no significant effect on bradykinin-induced, EDHF-mediated vasodilatation in this preparation. However, as expected (Beny & Schaad, 2000; Edwards *et al.*, 2000), the combination of charybdotoxin and apamin was required to block the EDHF response. Indeed, this combination virtually abolished the non-prostanoid, non-nitric oxide-mediated vasodilatation in this preparation, strengthening the view that it was mediated by EDHF.

In porcine coronary arteries that had been treated with ascorbate (150 μ M) the bradykinin-induced, EDHF-mediated vasodilatation was completely unaffected. This, clearly, is in stark contrast to the large inhibitory effect of a lower concentration of ascorbate (50 μ M) in the perfused bovine eye.

It is unclear why physiological concentrations of ascorbate inhibit EDHF-mediated vasodilatation in the bovine eye but not in the porcine coronary artery, but several

explanations are possible. For example, in the bovine eye EDHF-mediated vasodilatation is inhibited by charybdotoxin alone and the blockade of EDHF by ascorbate was similar in character: both caused an inhibition of vasodilatation and unmasked a vasoconstrictor response to acetylcholine (see Chapter 4 and Section 5.4). On this basis, it was possible that ascorbate could be inhibiting EDHF-mediated vasodilatation by blocking IK_{Ca} channels. It might therefore be expected that ascorbate alone would have no effect in the porcine coronary artery where blockade of both SK_{Ca} and IK_{Ca} channels is required to inhibit EDHF-mediated vasodilatation (Beny & Schaad, 2000; Edwards *et al.*, 2000). However, in the porcine coronary artery, ascorbate failed to inhibit the EDHF-mediated vasodilatation in combination with charybdotoxin and failed to enhance the small blocking effect of apamin. Thus, it appears that ascorbate does not block either SK_{Ca} or IK_{Ca} , at least not in the porcine coronary artery. Therefore, the suggestion that ascorbate may regulate K_{Ca} channel gating by reducing a critical disulphide bond seems unlikely (see Section 5.3). Whether there is heterogeneity between K_{Ca} channels in the bovine eye and porcine coronary artery is unknown at present. As already described (see Chapter 3 and Section 5.3) one way to investigate if an IK_{Ca} channel is the target for ascorbate in the bovine eye would be to examine the effects of this antioxidant on vasodilatation induced by an opener of IK_{Ca} channels. Unfortunately, however, this was not possible as the putative opener of IK_{Ca} channels, DCEBIO (Singh *et al.*, 2001), produced vasodilatation insensitive to blockade with charybdotoxin in the bovine eye.

Another explanation that could potentially account for the difference in the ability of ascorbate to inhibit EDHF-mediated vasodilatation in the bovine eye and porcine coronary artery is the size of the vessel studied. Specifically, the porcine coronary artery

is a large conduit artery, whereas the resistance blood vessels studied in the bovine eye are likely to be small arterioles. Indeed, vessel size is known to affect the nature of vasodilator responses in the rat mesenteric arterial bed; specifically, as the vessel diameter decreases the relative contribution of EDHF to the vasodilator responses increases and that of nitric oxide decreases (Hwa *et al.*, 1994). Furthermore, myoendothelial gap junctional plaque expression increases as vessel diameter decreases from the superior mesenteric artery to the third-order arteries (Sandow & Hill, 2000). Moreover, the effectiveness of inhibitors of gap junctional communication to block EDHF responses (Chaytor *et al.*, 1998; Chaytor *et al.*, 2001) increases with decreasing vessel size (Berman *et al.*, 2002; Chaytor *et al.*, 2001). Therefore, if ascorbate were inhibiting EDHF-mediated vasodilatation by interfering with gap-junctional communication it may be one explanation for its effectiveness in the perfused bovine eye but not in the porcine coronary artery.

Another difference between the perfused bovine eye and porcine coronary artery is that the ciliary body of the eye has the ability to concentrate ascorbate from blood plasma into the aqueous humour (Millar & Kaufman, 1995). This ability to concentrate ascorbate may result in high local concentrations of ascorbate in the eye and these high local concentrations could potentially underlie the inhibition of EDHF in the bovine eye. This argument seems unlikely, however, since EDHF-mediated vasodilatation is inhibited by ascorbate in the rat perfused mesenteric arterial bed (see Chapter 4 and Section 5.7), which lacks the ability to concentrate ascorbate. Nevertheless, the ascorbate-induced inhibition of EDHF in the rat mesenteric arterial bed has a far slower time course than in the bovine eye; therefore the high concentration of ascorbate in the eye could still aid inhibition of EDHF-mediated vasodilatation.

5.9 Ascorbate appears to inhibit EDHF-mediated vasodilatation in the ciliary vascular bed without the need for concentration by the ciliary body

As discussed in Section 5.8 it is possible that because the ciliary body of the bovine eye can actively transport ascorbate to levels of around 1 mM, the concentration of ascorbate that actually blocks EDHF-mediated vasodilatation may be considerably higher than the perfusate concentration (50 μ M). Consequently, experiments were conducted to assess whether preventing the accumulation of ascorbate in the bovine eye would affect the ability of this antioxidant to cause blockade of EDHF-mediated vasodilatation. In these experiments, the concentration of ascorbate in the aqueous and vitreous humours was measured using a spectrophotometric assay based on those described by (Lykkesfeldt *et al.*, 1995) and (Ortega-Barrales *et al.*, 1998). Using this assay the measured concentration of ascorbate in the aqueous and vitreous humours (1.1 ± 0.1 and 0.9 ± 0.1 mM, respectively) from freshly obtained eyes (within 1 hour of killing) was similar to those previously reported for the bovine eye (1.1 and 0.5 mM, respectively; Davson, 1980). Surprisingly, when the eyes were perfused for a 120 min period with Krebs solution containing no ascorbate, these concentrations of ascorbate were maintained. Paradoxically, the levels of ascorbate appeared to be slightly reduced when eyes were perfused with Krebs solution containing ascorbate (50 μ M); however, these falls in ascorbate concentration were not statistically significant and may represent the variation within the preparation. It would have been expected that during the 120 min study period, there would have been some consumption of ascorbate in redox reactions; however, the maintenance of the ascorbate concentration may reflect the ability of the epithelia of the cornea and iris/ciliary body (and hence the posterior and anterior chambers) to rapidly reduce any dehydroascorbate formed back to ascorbate

(Bode *et al.*, 1991; Bode *et al.*, 1993). Regardless of the mechanisms involved, the data suggest that the concentration of ascorbate in the ocular fluids is stringently controlled.

In experiments where the aqueous humour was continuously flushed by infusing Krebs solution into the anterior and posterior chambers, to prevent the accumulation of ascorbate, its concentration in the aqueous humour fell below detectable levels ($<10 \mu\text{M}$). The concentration of ascorbate in the vitreous humour was unaffected by this "flushing" procedure indicating that the "barrier" between the posterior and vitreous chambers remained intact. Furthermore, in control experiments the acetylcholine-induced, EDHF-mediated vasodilator responses were unaffected by flushing the posterior and anterior chamber chambers of the eye with normal Krebs solution. However, flushing also failed to prevent the blockade of EDHF-mediated vasodilatation when eyes were perfused with Krebs containing ascorbate ($50 \mu\text{M}$). Therefore, it appears likely that an intravascular ascorbate concentration of $50 \mu\text{M}$ truly is, itself, sufficient to block EDHF in the ciliary vascular bed of the bovine eye, and that further tissue concentration of this antioxidant is not necessary for blockade to occur.

Further evidence that concentration of ascorbate in aqueous humour is not required for the blockade of EDHF was provided by experiments where both the aqueous humour and vitreous humour were removed from the eye, resulting in a preparation where the ciliary circulation could be studied without the influence of either of the endogenous "reservoirs" of ascorbate, as well as preventing its accumulation. Under these conditions, when eyes were perfused with Krebs containing no ascorbate, the acetylcholine-induced, EDHF-mediated vasodilator responses were completely normal. In contrast, when ascorbate ($50 \mu\text{M}$) was included in the Krebs solution it still produced

the characteristic blockade of EDHF-mediated vasodilatation and uncovered a vasoconstrictor response. Thus, it would seem highly unlikely that accumulation of ascorbate in the aqueous or vitreous humour is a prerequisite for blockade of EDHF-mediated vasodilatation to occur. Therefore, it is likely that the ascorbate-induced blockade is mediated by the concentration that was infused, i.e. usually 50 μ M, which is the normal plasma concentration (Keaney & Vita, 1995; Levine *et al.*, 1996). This conclusion is further supported by the ability of ascorbate to inhibit EDHF-mediated vasodilatation in the mesenteric arterial bed of the rat, a preparation unable to concentrate ascorbate.

5.10 Future directions or studies

The evidence from the current study strongly indicates that the endothelium-dependent, acetylcholine- or bradykinin-induced vasodilator responses in the ciliary vascular bed of the bovine eye are mediated by EDHF. However, this conclusion is only based on measurements of perfusion pressure and to date, no electrophysiological recordings of membrane potential have been taken from the ciliary vascular bed. It would, therefore, be desirable for recordings of membrane potential from the ciliary vasculature of the bovine eye to be obtained to substantiate the inferred role for EDHF and to define the pharmacology of any hyperpolarization and its relationship to changes in smooth muscle tone. It may even be possible to infer changes in membrane potential in whole tissue by injecting voltage-sensitive dyes into the ocular blood vessels. Regardless, measurement of membrane potential may also give insights to the locus of the ascorbate-induced blockade of EDHF. For example, by impaling the appropriate cell type it might be possible to determine whether the blockade occurs at the level of the endothelium or smooth muscle.

The mechanism by which ascorbate inhibits EDHF-mediated responses in the ciliary vascular bed of the bovine eye is far from understood. The evidence suggests that the blockade of EDHF is highly selective and is due to an antioxidant action of ascorbate, perhaps by reducing a critical disulphide bond. However, the locus of the blockade also remains unclear; possible targets may include: K_{Ca} channels (Cai & Sauve, 1997; Edwards *et al.*, 1998; Zygmunt & Hogestatt, 1996), cytochrome P450 (Bauersachs *et al.*, 1994), and gap junctional communication (Chaytor *et al.*, 1998). The lack of effect of ascorbate on EDHF-mediated vasodilatation in the porcine coronary would appear to rule out the possibility that it inhibits EDHF by blocking a K_{Ca} channel. To date, the effect of blockade of cytochrome P450 or gap junctional communication has not been examined on EDHF-mediated vasodilatation in the bovine eye; if either contributes to the EDHF response it would be essential to examine the effects of ascorbate on their function.

Regardless of the mechanism of the ascorbate-induced blockade of EDHF-mediated vasodilatation in the bovine eye, it is also important to establish the physiological significance of such a process. For example this study has raised the possibility that ascorbate may act as a molecular "switch" where, under normal conditions, it may suppress EDHF-mediated vasodilatation, leading to predominance of the nitric oxide pathway. Consequently, it would be of interest to investigate if ascorbate could potentiate nitric oxide-dependent vasodilatation at the expense of EDHF in preparations in which both a nitric oxide and EDHF-mediated vasodilatation can be observed, such as the rat mesenteric arterial bed (McCulloch *et al.*, 1997).

In addition to ascorbate, there are many other endogenous antioxidants, not examined in this study, that have a protective role in vascular diseases (Halliwell & Gutteridge, 1989). For example, vitamin E deficiency is implicated in atherosclerosis (for review see; Carr *et al.*, 2000; Keaney & Vita, 1995) and acute supplementation of this vitamin can restore nitric oxide-mediated vasodilatation in a model of atherosclerosis in rabbits (Keaney *et al.*, 1993). Therefore, it would be prudent to conduct experiments to assess if vitamin E can mimic the effects of ascorbate on agonist-induced, EDHF-mediated vasodilatation.

In the porcine coronary artery ascorbate does not cause blockade of EDHF-mediated vasodilatation, hence it is unlikely that ascorbate blocks EDHF universally. Therefore differences in the nature of EDHF response between coronary and ocular vessels should be investigated. For example, EETs seem to play an important role in porcine coronary vessels (Fisslthaler *et al.*, 1999; Fisslthaler *et al.*, 2000; Popp *et al.*, 1996; Popp *et al.*, 2002) whereas in other vessels such as rat hepatic artery (Zygmunt *et al.*, 1996) and mesenteric artery (Edwards *et al.*, 1998) K^+ ions seem to account for the EDHF response. To date, ascorbate-induced blockade of EDHF-mediated vasodilatation has only been observed in perfused vascular beds (ciliary vascular bed of the bovine eye and rat mesenteric arterial bed). A major difference between the perfused vascular beds and the isolated coronary artery is the size of the vessels studied (resistance arteries and a conduit artery, respectively). Therefore, further investigation of the size of vessel studied on the ability of ascorbate to block EDHF-mediated vasodilatation should be undertaken. Furthermore, in rings of porcine coronary artery there is an absence of flow, therefore it would be interesting to investigate the effect of flow on the ascorbate-induced block of EDHF-mediated vasodilatation.

Finally, as nitric oxide synthase is located with the parasympathetic (nitroergic) nerves supplying the ocular vasculature in many species including humans (Tamm *et al.*, 1995), pigeons (Cuthbertson *et al.*, 1997), dogs (Toda *et al.*, 1994) and monkeys (Toda *et al.*, 1996), it is likely that nitric oxide released from these perivascular nerves contributes to vasomotor control in the eye. Indeed, as described in Section 1.3.1.2, nitric oxide mediates vasodilatation evoked by stimulation of perivascular nerves in the bovine ciliary artery (Weinkle *et al.*, 1994). As agonist-induced vasodilatation in the ciliary vascular bed is mediated entirely by EDHF, it would be interesting to characterise the contribution of neural release of nitric oxide to vasomotor control in this preparation.

5.11 Conclusions

The findings of this study demonstrate that in the ciliary vascular bed of the bovine eye, endothelium-dependent, agonist-induced vasodilator responses are mediated entirely by EDHF. These EDHF-mediated vasodilations are blocked in a time- and concentration-dependent manner by concentrations of ascorbate within the normal blood plasma range (30-150 μM). This blockade of EDHF-mediated vasodilatation is highly selective and appears to be due to an antioxidant action of ascorbate, possibly involving the reduction of a critical disulphide bond. Blockade of EDHF by ascorbate appears to be unrelated to the eye's ability to concentrate ascorbate as EDHF-mediated vasodilatation in the rat mesenteric arterial bed is also affected by this antioxidant. However, the finding that EDHF-mediated vasodilatation is unaffected by ascorbate in the porcine coronary artery indicates that ascorbate does not universally block EDHF. Thus, further work will be

necessary to characterise the mechanism and physiological relevance of the ability of ascorbate to block EDHF at discrete vascular sites.

Chapter 6

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